UNITED STATES ENVIRONMENTAL PROTECTION AGENCY
WASHINGTON, D.C. 20460

JUN - 2 2003

OFFICE OF PREVENTION, PESTICIDES AND TOXIC SUBSTANCES

SUBJECT: Transmittal of *Data Evaluation Records* for June Scientific Advisory Panel Meeting on Atrazine

FROM: Steven Bradbury, Director
Environmental Fate and Effects Division
Office of Pesticide Programs

TO: Paul Lewis,
Designated Federal Official,
FIFRA Scientific Advisory Panel

We have attached the Data Evaluation Records which provide the Agency's review of the studies discussed in the white paper for the June 17 - 20, 2003 Scientific Advisory Panel (SAP) meeting, "Potential Developmental Effects of Atrazine on Amphibians".

Thank you for your support. Feel free to call on us if you have any questions.

Attachments

cc: T. Steeger

Data Evaluation Records for the SAP Meeting on The Potential Developmental Effects of Atrazine Amphibians

DER #1 for Study #1: Smith, E., L DuPreez and K. Solomon. 2003. Field exposure of *Xenopus laevis* to atrazine and other triazines in South Africa: exposure characterization and assessment of laryngeal and gonadal responses. The Institute of Environmental & Human Health, Texas Tech University, Lubbock, Texas 79490 (USA) and School of Environmental Sciences and Development, Potchefstroom University for CHE, Private Bag X6001, Potchefstroom 2520 (South Africa). Sponsor: Syngenta Crop Protection, Inc., Laboratory Study ID ECORISK Number SA -01B.

DER #2 for Study #2: Jones, P. D., M. B. Murphy, M. Hecker, J. P. Giesy. 2003. Tissue Pesticide Residues and Histology of the Larynx and Gonads in Native Green Frogs (*Rana clamitans*) Collected from Agricultural Areas in Michigan: Hormone Analysis. Aquatic Toxicology Laboratory, Michigan State University, 218C National Food Safety and Toxicology Center, E. Lansing, MI. Sponsor: Syngenta Crop Protection, Inc. Laboratory Study ID: ECORISK Number MSU-02.

DER #3 for Study #3: Hecker, M., K. K. Coady, D. L. Villeneuve, M. B. Murphy, P. D. Jones and J. P. Giesy. 2003. A Pilot Study of Response of Larval *Rana clamitans* to Atrazine Exposure: Assessment of Metamorphosis and Gonadal and Laryngeal Morphology and Selected Hormones and Enzyme Activities. Aquatic Toxicology Laboratory, Michigan State University, National Food Safety and Toxicology Center, E. Lansing, MI. Sponsor: Syngenta Crop Protection, Inc., Laboratory Study ID ECORISK Number MSU-03.

DER #4 for Study #4: Hecker, M., K. K. Coady, D. L. Villeneuve, M. B. Murphy, P. D. Jones and J. P. Giesy. 2003. Response of *Xenopus laevis* to Atrazine Exposure: Assessment of the Mechanism of Action of Atrazine. Aquatic Toxicology Laboratory, Michigan State University, National Food Safety and Toxicology Center, E. Lansing, MI. Sponsor: Syngenta Crop Protection, Inc., Laboratory Study ID ECORISK Number MSU-04.

DER #5 for Study #5: Crabtree, C.; E. E. Smith; J. A. Carr. 2003. Histology of the gonads and analysis of hormone levels in the native bull frog (*Rana catesbiena*) collected from agricultural areas in southern Iowa: pilot project. The Institute of Environmental and Human Health, Texas Technical University, Lubbock, Texas. Sponsor: Syngenta Crop Protection, Inc. Laboratory Identification Number ECORISK Number TTU -02.

DER #6 for Study #6: Sepulveda, M. S. and T. S. Gross. 2003. Characterization of Atrazine Exposures and Potential Effects in Florida Ecosystems Dominated by Sugarcane Agriculture: A Reconnaissance Survey of Amphibians in South Florida for the Assessment of Potential Atrazine Effects. Department of Physiological Sciences, University of Florida, Caribbean Science Center, Gainesville, Florida. Sponsor: Syngenta Crop Protection, Inc. Study ID: ECORISK Number UFL-02.

DER #7 for Study #7: Goleman, W. L. and J. A. Carr. 2003. Response of larval *Xenopus laevis* to Atrazine Exposure: Assessment of Metamorphosis and Gonadal and Laryngeal Morphology. The Institute of Environmental and Human Health, Texas Tech University, Texas Tech University Health Sciences Center, Lubbock, Texas. Sponsor: Syngenta Crop Protection, Inc., Laboratory Study ID ECORISK Number TTU-01.

DER #8 for Study #8: Villeneuve, D. L., K. Coady, M. Hecker, M. B. Murphy, P. D. Jones and J. P. Giesy. 2003. Methods development for the study of mechanism of action of atrazine in adult and metamophosing *Xenopus laevis* and *Rana clamitans*: aromatase induction. Aquatic Toxicology Laboratory, Michigan State University, National Food Safety and Toxicology Center, E. Lansing, MI. Sponsor: Syngenta Crop Protection, Inc., Laboratory Study ID ECORISK Number MSU-01.

DER #9 for Study #9: Smith, E. E., L. DuPreez, and K. Solomon. 2003. Field exposure of Xenopus laevis to atrazine and other triazines in South Africa: feasibility study for site characterization and assessment of laryngeal and gonadal responses. The Institute of Environmental and Human Health, Texas Tech University, Lubbock, Texas (USA) and School of Environmental Sciences and Development, Potchefstroom University for CHE, Potchefstroom, South Africa. Sponsor: Syngenta Crop Protection, Inc., Laboratory Study ID: ECORISK Number SA-01A

DER #10 for Study #10: Smith, E. E., L. DuPreez, and K. Solomon. 2003. Gonadal and laryngeal responses to field exposure of *Xenopus laevis* to atrazine in areas of corn production in South Africa. The Institute of Environmental and Human Health, Texas Tech University, Lubbock, TX and School of Environmental Sciences and Development, Potchefstroom University for CHE, Potchefstroom, South Africa. Sponsor: Syngenta Crop Protection, Inc., Laboratory Study ID: ECORISK Number SA-01C.

DER #11 for Study #11: DuPreez, L. H., A. M. Jooste and K. R. Solomon. 2003. Exposure of *Xenopus laevis* larvae to different concentrations of atrazine in semi-natural microcosms. School of Environmental Sciences and Development, Zoology Department, Potchefstroom University fo CHE, Potchefstroom 2520, South Africa. Sponsor: Syngenta Crop Protection, Inc., Laboratory Study ID ECORISK Number SA-01-D.

DER #12 for Study #12: Giesy, J. P., M. Hecker, and P. D. Jones. 2003. South African Analytical Support – Hormone and Aromatase Analysis (SA- 01C). Aquatic Toxicology Laboratory, Michigan State University, National Food Safety and Toxicology Center, E. Lansing, MI. Sponsor: Syngenta Crop Protection, Inc., Laboratory Study ID ECORISK Number MSU-07

DER #13 for Document #1: Reeder, A. L., G. L. Foley, D. K. Nichols, L. G. Hansen, B. Wikoff, S. Faeh, J. Eisold, M. B. Wheeler, R. Warner, J. E. Murphy, and V. R. Beasley. 1998. Forms and Prevalence of Intersexuality and Effects of Environmental Contaminants on Sexuality in Cricket Frogs (*Acris crepitans*). Environmental Health Perspectives 106 (5): 261 - 266.

DER #14 for Document #2: Hayes, T. B., A. Collins, M. Lee, M. Mendoza, N. Noriega, A. A. Stuart, and A. Vonk. 2002*a*. Hermaphroditic, demasculinized frogs after exposure to the herbicide atrazine at low ecologically relevant doses. Proceedings of the National Academy of Sciences 99 (8): 5476 - 5480.

DER #15 for Document #5: Hayes, T., K. Haston, M. Tsui, A. Hoang, C. Haeffele and A. Vonk. 2002*b*. Atrazine-induced hermaphroditism at 0.1 ppb in American leopard frogs (*Rana pipiens*): laboratory and field evidence. Environmental Health Perspectives 111 (4): 568 - 575.

DER #16 for Document #6: Tavera-Mendoza, L., S. Ruby, P. Brousseau, M. Fournier, D. Cyr and D. Marcogliese. 2001. Response of the amphibian tadpole *Xenopus laevis* to atrazine during sexual differentiation of the ovary. Environmental Toxicology and Chemistry 21 (6): 1264 - 1267.

DER #17 for Document #7: Tavera-Mendoza, L., S. Ruby, P. Brousseau, M. Fournier, D. Cyr and D. Marcogliese. 2001. Response of the amphibian tadpole *Xenopus laevis* to atrazine during sexual differentiation of the testes. Environmental Toxicology and Chemistry 21 (3): 527 - 531.

Data Evaluation Report on Field Exposure of *Xenopus laevis* to Atrazine and Other Triazines in South Africa: Exposure Characterization and Assessment of Laryngeal and Gonadal Responses

EPA MRID Number 458677-01

Data Requirement:

EPA DP Barcode	D288775
EPA MRID	458677-01
EPA Guideline	70-1(Special Study)

Test material: **Purity:** not reported

Common name Atrazine
chemical name: IUPAC
CAS name 6-chloro-N-ethyl-N'-(1-methylethyl)-1,3,5-triazine-2,4-diamine
CAS No. 1912-24-9
synonyms
EPA PC Code: 80803

Thomas M Steeger

Primary Reviewer: Thomas M. Steeger, Ph.D., Senior Biologist **Date:** April 9, 2003
Environmental Fate and Effects Division, ERB 4, U. S. Environmental Protection Agency

Joseph E. Tietge

Secondary Reviewer(s): Joseph E. Tietge, M.S., Research Aquatic Biologist **Date:** 4/16/03
Mid-Continent Ecology Division, National Health and Environmental Effects Research Laboratory (Duluth), U. S. Environmental Protection Agency

Stephanie R Irene

Stephanie Irene, Ph.D., Senior Advisor **Date:** 4/29/03
Environmental Fate and Effects Division, ERB 3, U. S. Environmental Protection Agency

Mary Frankenberry

Mary J. Frankenberry, Senior Statistician **Date:** 4/28/03
Environmental Fate and Effects Division, ERB 3, U. S. Environmental Protection Agency

EPA PC Code 080803

Date Evaluation Completed: 06/01/2003

CITATION: Smith, E., L DuPreez and K. Solomon. 2003. Field exposure of *Xenopus laevis* to atrazine and other triazines in South Africa: exposure characterization and assessment of laryngeal and gonadal responses. The Institute of Environmental & Human Health, Texas Tech University, Lubbock, Texas 79490 (USA) and School of Environmental Sciences and Development, Potchefstroom University for CHE, Private Bag X6001, Potchefstroom 2520 (South Africa). Sponsor: Syngenta Crop Protection, Inc., Laboratory Study ID ECORISK Number SA-01B

EXECUTIVE SUMMARY:

This study is the second phase of a project to assess the possible effects of exposure to atrazine and other triazines on African clawed frogs (*Xenopus laevis*) in their South African native range under field conditions. The objective of this phase of the study was to characterize the exposure of frog populations to atrazine and related triazines in surface waters of both reference (no corn/no atrazine-triazine use) and experimental (corn growing/atrazine-triazine use) ponds from November 2001 through early June 2002. During the second two months of the study, rainfall was roughly double the 10-year average and resulted in all of the earthen ponds overflowing their banks for as long as two months. Based on EFED's analysis of raw residue data, maximum atrazine residues in experimental ponds over the study period ranged from 1.46 to 11.6 ug/L while reference ponds ranged from 0.41 to 1.62 ug/L. However, the atrazine degradate diaminochlorotriazine (DACT) maximum residues ranged from 4.6 to 8.2 ug/L at experimental sites and form 6.8 to 7.4 ug/L at reference sites. Two other atrazine degradates, i.e., desethylated atrazine (DEA)and desisopropyl atrazine (DIA) showed roughly similar maximum residue levels in both experimental and reference sites. Maximum residues of terbuthylazine ranged from 1.8 to 5.3 ug/Lat experimental sites and from 2.4 to 2.8 ug/L at reference sites. According to the report, triazine residues in the reference ponds were likely due to wind effect; the authors speculate that the high rain events during the sampling period likely reduced atrazine levels and that "frogs living in these dams [ponds] were undoubtedly exposed to much higher atrazine and other triazine levels than had been recorded during the present study." Given that atrazine and/or its degradates were present in reference ponds at levels at times equivalent to some experimental pond sites and the authors concede that atrazine exposure prior to the winter floods was likely higher at all sites, it is unclear how the study can differentiate atrazine effects on frogs at reference and experimental sites. The high variability in exposure could potentially confound any attempt to document significant differences in effects.

The objective of this study was to examine the effects of atrazine on *X. laevis* in its native habitat (South Africa). Initially the study was intended to test whether morphological and biochemical differences existed between clawed frogs in atrazine-exposed (experimental) versus non-exposed (reference) ponds. The criteria for differentiating reference and experimental sites included production of corn and use of atrazine in the vicinity, plus the presence of *X. laevis* in a pond. Based on an initial survey of the sampling area, five experimental (atrazine exposure) and three reference (no atrazine exposure) sites were selected (458677-09). However, subsequent sampling during later phases of the study revealed that the reference sites all contained measurable residues of atrazine, its degradates, and terbutyhylazine (triazine herbicide not registered for use in the USA) that were, in some cases, higher than sites considered representative of atrazine exposure

I. MATERIALS AND METHODS

GUIDELINE FOLLOWED: Nonguideline Study
COMPLIANCE: Not conducted under full GLP; however, most practices as defined by 40 CFR Part 160, August 19, 1989 were established for this study, including but not limited to:

- Written, authorized protocol
- Written, authorized Standard Operating Procedures for all key procedures.
- Organization and Personnel were sufficient in terms of number, education, training and experience.
- Facilities were of suitable size and construction
- Equipment used was of appropriate design and adequate capacity.
- Test material identity, strength, purity and composition were characterized.
- Independent QA Inspections were conducted.
- Final report was written
- Raw data, documentation, records, protocols, and final report were archived.

A. MATERIALS:

1. Test Material Atrazine

Description: Not reported

Lot No./Batch No. : Not reported

Purity: Not reported
Stability of Compound Under Test Conditions: Not reported

Storage conditions of test chemicals: Not reported

2. Test organism:

Species: African clawed frog (*Xenopus laevis*)

Age at test initiation: Adults
Weight at study initiation: (mean and range) not reported
Length at study initiation: (mean and range) not reported

Source: Adult *X. laevis* were field collected in two areas (3 non-corn growing areas and 5 corn growing areas) in the vicinity of Potchefstrooom, South Africa, using traps baited with liver and meat scraps.

B. STUDY DESIGN:

Objective: 1. To characterize the exposure of populations of *Xenopus laevis* larvae, metamorphs and adults to atrazine and related triazines in surface water in reference (no corn production or atrazine/triazine use) and exposed habitats in the proximity of corn production and atrazine/triazine use in the Potchestroom region of South Africa.

1. Experimental Conditions

A total of 8 sampling sites in two adjacent regions (5 in Viljoenskroon corn growing region = E; 3 in non-corn growing Potchefstroom region = C) in South Africa. Experimental site selection based on proximity of corn, previous and/or planned use of atrazine and terbuthylazine, and presence of *Xenopus*. Reference sites based on absence of corn production, absence of triazine amd terbuthylazine in the water, and presence of *Xenopus*.

Biweekly water/sediment samples collected from early November 2001 to early June 2002. Within 5 hours of collection water samples kept at 4°C and transported to testing lab within 24 hours of collection. Water temperature, conductivity, dissolved oxygen and pH were recorded at reference points on each sampling site.

Pesticide and metabolite determinations in water conducted at Department of Microbiology of the School of Environmental Sciences and Development at the Potchefstroom University. Quality control conducted at CSIR (Pretoria, South Africa) and SGS (Midrand, South Africa).

Climatological conditions characterized in terms of air temperature and rainfall.

Agricultural practices in each of the study site catchment areas characterized as to crop, stage of crop development, *e.g.*, just planted, in flower *etc.*, and pesticides applied..

Compounds of interest included atrazine, its metabolites desethylated atrazine (DEA), desisopropyl atrazine (DIA), diaminochlorotriazine (DACT), and terbuthylazine plus simazine and acetochlor.

Non-corn growing sites had secchi disc readings ranging from 6.5 to 32 cm; pH ranged from 5.1 to 8.8; some of the control ponds were subject to drying (semi-permanent).

Corn-growing sites had pond surface areas ranging from 2,400 m^2 to 68,000 m^2; pH ranged from 7.2 - 10.8 and secchi disc readings ranging from 6.5 to 207 cm.

II. RESULTS and DISCUSSION: [All results discussed in this section and the next are those reported by the study authors. Although supplemental data are typically used in a qualitative manner only, EFED verified spreadsheet data and ran basic statistical analyses on the major study parameters. See attached appendix. If results differed in any substantive way, the difference was reported in the text below.]

Rainfall during November and December of sampling period were more than double (~ 150 to 200 mm) the long term average of approximately 100 mm precipitation. Air temperature over the study period were relatively consistent with 10-year minimums and maximums. High rainfall in November and December resulted in the majority of the area being planted in corn late in the season. Although corn is typically planted in November, the high rains delayed planting until the first week of January. Because of the heavy rains, all ponds overflowed their embankments until early January.

The following insecticides were used in the study's catchment areas: terbufos (79.2 to 460 g/ha), cypermethrin (6.6 g/100 m row), monocrotophos (1.5 L/ha). Herbicides included acetochlor, atrazine, terbuthylazine, S-metolaclhor, simazine, cyanazine and dimthenomid.

No herbicides were detected in any of the sediment samples analyzed. The level of detection for water samples ranged from 0.5 to 1.0 µg/L. The report records values of <0.6 µg/L as 0.25 µg/L.

Table 1 presents maximum residues of atrazine and its degradates DACT, DEA and DIA; while maximum atrazine residues were roughly an order of magnitude higher at experimental sites, reference sites had detectable residues of atrazine. Maximum residues of DACT, DEA and DIA were similar in both reference and experimental sites. Both DEA and DACT had peak residues in reference ponds that were generally higher than all of the experimental pond sites except E8. The highest residues of DACT in reference ponds occurred in early December, early to mid-February and mid- to late May; a relatively similar pattern occurred in experimental ponds. Additionally, terbuthylazine in reference pond C6 tended to remained relatively constant at around 1 µg/L until March when it spiked to over 2 µg/L; only experimental ponds E1, E6 and E8 had monthly terbuthylazine residues higher than reference pond C6. While the high rain events in November and December may have explained potential triazine contamination of reference sites, the spikes in February and May suggest that triazine contamination of reference sites was recurrent and may not have been associated with unusual weather. According to the report though, triazine residues in the reference ponds were likely due to wind effect and the authors speculate that the high rain events during the sampling period likely reduced atrazine levels and that "frogs living in these dams were undoubtedly exposed to much higher atrazine and other triazine levels than had been recorded during the present study.

Reference ponds contained soft water (total hardness range 18 - 25 mg/L as CaCO3) while experimental ponds all contained moderately hard water (total hardness range: 182 - 200 mg/L). Lead residues ranged form 0.08 to 0.09 mg/L in reference ponds while it was nondetectable in experimental sites. Reference pond also seemed to be an outlier in terms of the amount of siltation; while all other ponds (reference and experimental) had silica levels ranging from 4.3 - 8.7 mg/L, reference pond C6 contained 49.2 mg/L. Additionally, pond C6 had the highest chromium (100.5 mg/Kg) and titanium (0.6 mg/L) residues

Table 1. Maximum residues of atrazine, diaminochlorotriazine (DACT), desethylated atrazine (DEA), desisopropyl atrazine (DIA), terbuthylazine, simazine and acetochlor detected in surface water collected from reference sites (no corn grown) and experimental sites (corn grown) from November 2001 to March 2002.

Residue	Reference Sites (C1, C3, and C6) µg/L	Experimental Sites (E1, E3, E4, E6, and E8) µg/L
Atrazine	0.41 - 1.62	1.46 - 11.6
DACT	6.83 - 7.38	4.59 - 8.16
DEA	0.38 - 2.21	0.57 - 1.9
DIA	0.45 - 1.34	0.69 - 0.93
Terbuthylazine	2.39 - 2.79	1.82 - 5.30
Simizine	0.25 - 0.25	0.25 - 3.10

Acetochlor	0.25 - 0.25	0.25 - 1.0

E. STUDY DEFICIENCIES:

Atrazine, its degradates and terbuthylazine are present in reference sites. Study failed to provide data on other pesticides.

F. REVIEWER'S COMMENTS:

This study is the second phase of a project to assess the possible effects of exposure to atrazine and other triazines on African clawed frogs (*Xenopus laevis*) in their South African native range under field conditions. The objective of this phase of the study was to characterize the exposure of frog populations to atrazine and related triazines in surface waters of both reference (no corn/no atrazine-triazine use) and experimental (corn growing/atrazine-triazine use) ponds from November 2001 through early June 2002. During the second two months of the study, rainfall was roughly double the 10-year average and resulted in all of the earthen ponds overflowing their banks for as long as two months. Based on EFED's analysis of raw residue data, maximum atrazine residues in experimental ponds over the study period ranged from 1.46 to 11.6 ug/L while reference ponds ranged from 0.41 to 1.62 ug/L. However, the atrazine degradate diaminochlorotriazine (DACT) maximum residues ranged from 4.6 to 8.2 ug/L at experimental sites and form 6.8 to 7.4 ug/L at reference sites. Two other atrazine degradates, i.e., desethylated atrazine (DEA)and desisopropyl atrazine (DIA) showed roughly similar maximum residue levels in both experimental and reference sites. Maximum residues of terbuthylazine ranged from 1.8 to 5.3 ug/Lat experimental sites and from 2.4 to 2.8 ug/L at reference sites. According to the report, triazine residues in the reference ponds were likely due to wind effect; the authors speculate that the high rain events during the sampling period likely reduced atrazine levels and that "frogs living in these dams [ponds] were undoubtedly exposed to much higher atrazine and other triazine levels than had been recorded during the present study." Given that atrazine and/or its degradates were present in reference ponds at levels at times equivalent to some experimental pond sites and the authors concede that atrazine exposure prior to the winter floods was likely higher at all sites, it is unclear how the study can differentiate atrazine effects on frogs at reference and experimental sites. The high variability in exposure could potentially confound any attempt to document significant differences in effects.

G. CONCLUSIONS:

This study is the second phase of a project to assess the possible effects of exposure to atrazine and other triazines on African clawed frogs (*Xenopus laevis*) in their South African native range under field conditions. The objective of this phase of the study was to characterize the exposure of frog populations to atrazine and related triazines in surface waters of both reference (no corn/no atrazine-triazine use) and experimental (corn growing/atrazine-triazine use) ponds from November 2001 through early June 2002. During the second two months of the study, rainfall was roughly double the 10-year average and resulted in all of the earthen ponds overflowing their banks for as long as two months. Based on EFED's analysis of raw residue data, maximum atrazine residues in experimental ponds over the study period ranged from 1.46 to 11.6 ug/L while reference ponds ranged from 0.41 to 1.62 ug/L. However, the atrazine degradate diaminochlorotriazine (DACT) maximum residues ranged from 4.6 to 8.2 ug/L at experimental sites and form 6.8 to 7.4 ug/L at reference sites. Two other atrazine degradates, i.e., desethylated atrazine (DEA)and desisopropyl atrazine (DIA) showed roughly similar maximum residue levels in both experimental and reference sites. Maximum residues of terbuthylazine ranged from 1.8 to 5.3 ug/Lat experimental sites and from 2.4 to 2.8 ug/L at reference sites. According to the report, triazine residues in the reference ponds were likely due to wind effect; the authors speculate that the high rain events during the sampling period likely reduced atrazine levels and that "frogs living in these dams [ponds] were undoubtedly exposed to much higher atrazine and other triazine levels than had been recorded during the present study."

Given that atrazine and/or its degradates were present in reference ponds at levels at times equivalent to some experimental pond sites and the authors concede that atrazine exposure prior to the winter floods was likely higher at all sites, it is unclear how the study can differentiate atrazine effects on frogs at reference and experimental sites. The high variability in exposure could potentially confound any attempt to document significant differences in effects.

ANOVA FOR CHEMICAL RESIDUES ACROSS SAMPLING AREAS 51

Obs	SITE	RESIDUE	TREAT	_TYPE_	_FREQ_	MEAN	STD	MIN	MAX
1	E1	ATRA	CORN GROW	0	24	3.07583	1.82021	0.05	6.78
2	E1	DACT	CORN GROW	0	24	0.94542	1.64876	0.05	5.20
3	E1	DEA	CORN GROW	0	24	0.68417	0.46684	0.05	1.66
4	E1	DIA	CORN GROW	0	24	0.39042	0.24017	0.05	0.93
5	E1	TERB	CORN GROW	0	24	2.84875	1.46945	0.05	5.30
6	E3	ATRA	CORN GROW	0	24	0.79667	0.32979	0.14	1.46
7	E3	DACT	CORN GROW	0	24	1.07333	1.81710	0.05	5.96
8	E3	DEA	CORN GROW	0	24	0.30375	0.16691	0.05	0.64
9	E3	DIA	CORN GROW	0	24	0.25375	0.19147	0.05	0.81
10	E3	TERB	CORN GROW	0	24	0.87667	0.79778	0.05	3.31
11	E4	ATRA	CORN GROW	0	24	0.56333	0.43697	0.15	1.96
12	E4	DACT	CORN GROW	0	24	1.04875	1.51311	0.05	4.59
13	E4	DEA	CORN GROW	0	24	0.19333	0.11698	0.05	0.57
14	E4	DIA	CORN GROW	0	24	0.23333	0.18647	0.05	0.69
15	E4	TERB	CORN GROW	0	24	0.56250	0.60623	0.05	1.82
16	E6	ATRA	CORN GROW	0	24	2.19500	1.39259	0.05	4.46
17	E6	DACT	CORN GROW	0	24	1.46625	2.01606	0.05	6.40
18	E6	DEA	CORN GROW	0	24	0.51542	0.46881	0.05	1.90
19	E6	DIA	CORN GROW	0	24	0.27875	0.22601	0.05	0.86
20	E6	TERB	CORN GROW	0	24	1.77917	1.17789	0.05	4.43
21	E8	ATRA	CORN GROW	0	24	3.33875	2.58250	0.05	11.60
22	E8	DACT	CORN GROW	0	24	1.40375	2.34236	0.05	8.16
23	E8	DEA	CORN GROW	0	24	0.45875	0.37055	0.05	1.25
24	E8	DIA	CORN GROW	0	24	0.40208	0.23805	0.05	0.88
25	E8	TERB	CORN GROW	0	24	1.61542	1.12659	0.05	3.48
26	R1	ATRA	REFERENCE	0	24	0.24542	0.10496	0.05	0.41
27	R1	DACT	REFERENCE	0	24	1.01792	1.67744	0.05	6.88
28	R1	DEA	REFERENCE	0	24	0.12958	0.12743	0.05	0.60
29	R1	DIA	REFERENCE	0	24	0.19958	0.11845	0.05	0.47
30	R1	TERB	REFERENCE	0	24	0.32333	0.64632	0.05	2.11
31	R3	ATRA	REFERENCE	0	24	0.18042	0.15502	0.05	0.57
32	R3	DACT	REFERENCE	0	24	1.39167	1.90289	0.05	7.38
33	R3	DEA	REFERENCE	0	24	0.26333	0.56395	0.05	2.21
34	R3	DIA	REFERENCE	0	24	0.24000	0.27233	0.05	1.34
35	R3	TERB	REFERENCE	0	24	0.55708	0.88616	0.05	2.79
36	R6	ATRA	REFERENCE	0	24	0.24083	0.16981	0.05	0.69
37	R6	DACT	REFERENCE	0	24	1.17708	1.69010	0.05	6.82
38	R6	DEA	REFERENCE	0	24	0.10000	0.08038	0.05	0.38
39	R6	DIA	REFERENCE	0	24	0.16542	0.09385	0.05	0.45
40	R6	TERB	REFERENCE	0	24	1.08042	0.50886	0.48	2.67

AVERAGE CHEMICAL RESIDUES IN CORN-GROWING AND REFERENCE SITES IN SOUTH AFRICA 52

Obs	TREAT	RESIDUE	_TYPE_	_FREQ_	MEAN	STD	MIN	MAX
1	CORN GROW	ATRA	0	5	1.99392	1.27471	0.56333	3.33875
2	CORN GROW	DACT	0	5	1.18750	0.23203	0.94542	1.46625
3	CORN GROW	DEA	0	5	0.43108	0.19018	0.19333	0.68417
4	CORN GROW	DIA	0	5	0.31167	0.07898	0.23333	0.40208
5	CORN GROW	TERB	0	5	1.53650	0.89039	0.56250	2.84875
6	REFERENCE	ATRA	0	3	0.22222	0.03628	0.18042	0.24542
7	REFERENCE	DACT	0	3	1.19556	0.18756	1.01792	1.39167
8	REFERENCE	DEA	0	3	0.16431	0.08703	0.10000	0.26333
9	REFERENCE	DIA	0	3	0.20167	0.03734	0.16542	0.24000
10	REFERENCE	TERB	0	3	0.65361	0.38766	0.32333	1.08042

NONPARAMETRIC COMPARISON OF CHEMICAL RESIDUES ACROSS SAMPLING SITES 53

-- RESIDUE=ATRA --

The NPAR1WAY Procedure

Wilcoxon Scores (Rank Sums) for Variable UG
Classified by Variable SITE

SITE	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
E1	24	3463.00	2316.0	254.513858	144.291667
E3	24	2665.00	2316.0	254.513858	111.041667
E4	24	2085.00	2316.0	254.513858	86.875000
E6	24	3345.00	2316.0	254.513858	139.375000
E8	24	3504.00	2316.0	254.513858	146.000000
R1	24	1313.50	2316.0	254.513858	54.729167
R3	24	934.00	2316.0	254.513858	38.916667
R6	24	1218.50	2316.0	254.513858	50.770833

Average scores were used for ties.

Kruskal-Wallis Test

Chi-Square	109.1485
DF	7
Pr > Chi-Square	<.0001

Median Scores (Number of Points Above Median) for Variable UG
Classified by Variable SITE

SITE	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
E1	24	21.0	12.0	2.297278	0.875000
E3	24	19.0	12.0	2.297278	0.791667
E4	24	9.0	12.0	2.297278	0.375000
E6	24	22.0	12.0	2.297278	0.916667
E8	24	21.0	12.0	2.297278	0.875000
R1	24	0.0	12.0	2.297278	0.000000
R3	24	1.0	12.0	2.297278	0.041667
R6	24	3.0	12.0	2.297278	0.125000

Average scores were used for ties.

Median One-Way Analysis

Chi-Square	110.4219
DF	7
Pr > Chi-Square	<.0001

-- RESIDUE=DACT --

The NPAR1WAY Procedure

Wilcoxon Scores (Rank Sums) for Variable UG
Classified by Variable SITE

SITE	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
E1	24	2130.00	2316.0	243.756869	88.75000
E3	24	2220.00	2316.0	243.756869	92.50000
E4	24	2202.00	2316.0	243.756869	91.75000
E6	24	2449.50	2316.0	243.756869	102.06250
E8	24	2430.00	2316.0	243.756869	101.25000
R1	24	2238.00	2316.0	243.756869	93.25000
R3	24	2473.50	2316.0	243.756869	103.06250
R6	24	2385.00	2316.0	243.756869	99.37500

Average scores were used for ties.

Kruskal-Wallis Test

Chi-Square	1.8154
DF	7
Pr > Chi-Square	0.9693

Median Scores (Number of Points Above Median) for Variable UG
Classified by Variable SITE

SITE	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
E1	24	11.0	12.0	2.297278	0.458333
E3	24	11.0	12.0	2.297278	0.458333
E4	24	12.0	12.0	2.297278	0.500000
E6	24	12.0	12.0	2.297278	0.500000
E8	24	14.0	12.0	2.297278	0.583333
R1	24	11.0	12.0	2.297278	0.458333
R3	24	13.0	12.0	2.297278	0.541667
R6	24	12.0	12.0	2.297278	0.500000

Average scores were used for ties.

Median One-Way Analysis

Chi-Square	1.3264
DF	7
Pr > Chi-Square	0.9877

-- RESIDUE=DEA --

The NPAR1WAY Procedure

Wilcoxon Scores (Rank Sums) for Variable UG
Classified by Variable SITE

SITE	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
E1	24	3596.50	2316.0	251.896139	149.854167
E3	24	2741.00	2316.0	251.896139	114.208333
E4	24	2145.00	2316.0	251.896139	89.375000
E6	24	2974.00	2316.0	251.896139	123.916667
E8	24	2979.00	2316.0	251.896139	124.125000
R1	24	1435.50	2316.0	251.896139	59.812500
R3	24	1449.00	2316.0	251.896139	60.375000
R6	24	1208.00	2316.0	251.896139	50.333333

Average scores were used for ties.

Kruskal-Wallis Test

Chi-Square	75.5240
DF	7
Pr > Chi-Square	<.0001

Median Scores (Number of Points Above Median) for Variable UG
Classified by Variable SITE

SITE	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
E1	24	23.000000	12.0	2.277249	0.958333
E3	24	17.166667	12.0	2.277249	0.715278
E4	24	11.166667	12.0	2.277249	0.465278
E6	24	17.166667	12.0	2.277249	0.715278
E8	24	17.166667	12.0	2.277249	0.715278
R1	24	4.333333	12.0	2.277249	0.180556
R3	24	3.000000	12.0	2.277249	0.125000
R6	24	3.000000	12.0	2.277249	0.125000

Average scores were used for ties.

Median One-Way Analysis

Chi-Square	71.2969
DF	7
Pr > Chi-Square	<.0001

-- RESIDUE=DIA --

The NPAR1WAY Procedure

Wilcoxon Scores (Rank Sums) for Variable UG
Classified by Variable SITE

SITE	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
E1	24	3075.50	2316.0	254.029783	128.145833
E3	24	2274.50	2316.0	254.029783	94.770833
E4	24	2097.50	2316.0	254.029783	87.395833
E6	24	2326.50	2316.0	254.029783	96.937500
E8	24	3114.50	2316.0	254.029783	129.770833
R1	24	1983.00	2316.0	254.029783	82.625000
R3	24	1967.00	2316.0	254.029783	81.958333
R6	24	1689.50	2316.0	254.029783	70.395833

Average scores were used for ties.

Kruskal-Wallis Test

Chi-Square	25.6164
DF	7
Pr > Chi-Square	0.0006

The NPAR1WAY Procedure

Median Scores (Number of Points Above Median) for Variable UG
Classified by Variable SITE

SITE	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
E1	24	18.00	12.0	2.261098	0.750000
E3	24	11.50	12.0	2.261098	0.479167
E4	24	10.50	12.0	2.261098	0.437500
E6	24	11.50	12.0	2.261098	0.479167
E8	24	18.00	12.0	2.261098	0.750000
R1	24	10.50	12.0	2.261098	0.437500
R3	24	9.50	12.0	2.261098	0.395833
R6	24	6.50	12.0	2.261098	0.270833

Average scores were used for ties.

Median One-Way Analysis

Chi-Square	19.4252
DF	7
Pr > Chi-Square	0.0070

-- RESIDUE=TERB --

The NPAR1WAY Procedure

Wilcoxon Scores (Rank Sums) for Variable UG
Classified by Variable SITE

SITE	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
E1	24	3715.50	2316.0	252.107852	154.812500
E3	24	2158.50	2316.0	252.107852	89.937500
E4	24	1664.00	2316.0	252.107852	69.333333
E6	24	3049.50	2316.0	252.107852	127.062500
E8	24	2890.50	2316.0	252.107852	120.437500
R1	24	1112.00	2316.0	252.107852	46.333333
R3	24	1395.50	2316.0	252.107852	58.145833
R6	24	2542.50	2316.0	252.107852	105.937500

Average scores were used for ties.

Kruskal-Wallis Test

Chi-Square	77.4362
DF	7
Pr > Chi-Square	<.0001

Median Scores (Number of Points Above Median) for Variable UG
Classified by Variable SITE

SITE	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
E1	24	21.0	12.0	2.297278	0.875000
E3	24	11.0	12.0	2.297278	0.458333
E4	24	7.0	12.0	2.297278	0.291667
E6	24	18.0	12.0	2.297278	0.750000
E8	24	16.0	12.0	2.297278	0.666667
R1	24	3.0	12.0	2.297278	0.125000
R3	24	6.0	12.0	2.297278	0.250000
R6	24	14.0	12.0	2.297278	0.583333

Average scores were used for ties.

Median One-Way Analysis

Chi-Square	46.4236
DF	7
Pr > Chi-Square	<.0001

ANOVA FOR CHEMICAL RESIDUES ACROSS SAMPLING AREAS 63

-- RESIDUE=ATRA --

The ANOVA Procedure

Class Level Information

Class	Levels	Values
SITE	8	E1 E3 E4 E6 E8 R1 R3 R6

Number of observations 192

Dependent Variable: UG

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	7	297.3024703	42.4717815	27.66	<.0001
Error	184	282.5627875	1.5356673		
Corrected Total	191	579.8652578			

R-Square	Coeff Var	Root MSE	UG Mean
0.512710	93.20732	1.239220	1.329531

Source	DF	Anova SS	Mean Square	F Value	Pr > F
SITE	7	297.3024703	42.4717815	27.66	<.0001

Levene's Test for Homogeneity of UG Variance
ANOVA of Squared Deviations from Group Means

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
SITE	7	890.5	127.2	4.85	<.0001
Error	184	4830.0	26.2501		

Bartlett's Test for Homogeneity of UG Variance

Source	DF	Chi-Square	Pr > ChiSq
SITE	7	347.0	<.0001

```
                         Bonferroni (Dunn) t Tests for UG

NOTE: This test controls the Type I experimentwise error rate, but it generally has a higher
                          Type II error rate than REGWQ.


                     Alpha                               0.05
                     Error Degrees of Freedom             184
                     Error Mean Square               1.535667
                     Critical Value of t              3.17001
                     Minimum Significant Difference     1.134


              Means with the same letter are not significantly different.


                  Bon Grouping          Mean      N    SITE

                             A        3.3388     24    E8
                             A
                        B    A        3.0758     24    E1
                        B
                        B             2.1950     24    E6

                             C        0.7967     24    E3
                             C
                             C        0.5633     24    E4
                             C
                             C        0.2454     24    R1
                             C
                             C        0.2408     24    R6
                             C
                             C        0.1804     24    R3
```

ANOVA FOR CHEMICAL RESIDUES ACROSS SAMPLING AREAS 67

-- RESIDUE=DACT --

The ANOVA Procedure

Class Level Information

Class	Levels	Values
SITE	8	E1 E3 E4 E6 E8 R1 R3 R6

Number of observations 192

Dependent Variable: UG

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	7	6.8600062	0.9800009	0.29	0.9577
Error	184	624.5005417	3.3940247		
Corrected Total	191	631.3605479			

R-Square	Coeff Var	Root MSE	UG Mean
0.010865	154.7464	1.842288	1.190521

Source	DF	Anova SS	Mean Square	F Value	Pr > F
SITE	7	6.86000625	0.98000089	0.29	0.9577

Levene's Test for Homogeneity of UG Variance
ANOVA of Squared Deviations from Group Means

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
SITE	7	158.5	22.6424	0.45	0.8688
Error	184	9242.4	50.2302		

Bartlett's Test for Homogeneity of UG Variance

Source	DF	Chi-Square	Pr > ChiSq
SITE	7	6.2865	0.5067

-- RESIDUE=DACT --

The ANOVA Procedure

Bonferroni (Dunn) t Tests for UG

NOTE: This test controls the Type I experimentwise error rate, but it generally has a higher Type II error rate than REGWQ.

Alpha	0.05
Error Degrees of Freedom	184
Error Mean Square	3.394025
Critical Value of t	3.17001
Minimum Significant Difference	1.6859

Means with the same letter are not significantly different.

Bon Grouping	Mean	N	SITE
A	1.4663	24	E6
A			
A	1.4038	24	E8
A			
A	1.3917	24	R3
A			
A	1.1771	24	R6
A			
A	1.0733	24	E3
A			
A	1.0488	24	E4
A			
A	1.0179	24	R1
A			
A	0.9454	24	E1

ANOVA FOR CHEMICAL RESIDUES ACROSS SAMPLING AREAS 71

-- RESIDUE=DEA --

The ANOVA Procedure

Class Level Information

Class	Levels	Values
SITE	8	E1 E3 E4 E6 E8 R1 R3 R6

Number of observations 192

Dependent Variable: UG

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	7	7.03822500	1.00546071	8.40	<.0001
Error	184	22.01816667	0.11966395		
Corrected Total	191	29.05639167			

R-Square	Coeff Var	Root MSE	UG Mean
0.242226	104.4958	0.345925	0.331042

Source	DF	Anova SS	Mean Square	F Value	Pr > F
SITE	7	7.03822500	1.00546071	8.40	<.0001

Levene's Test for Homogeneity of UG Variance
ANOVA of Squared Deviations from Group Means

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
SITE	7	2.2597	0.3228	2.39	0.0233
Error	184	24.8825	0.1352		

Bartlett's Test for Homogeneity of UG Variance

Source	DF	Chi-Square	Pr > ChiSq
SITE	7	140.8	<.0001

ANOVA FOR CHEMICAL RESIDUES ACROSS SAMPLING AREAS 74

-- RESIDUE=DEA --

The ANOVA Procedure

Bonferroni (Dunn) t Tests for UG

NOTE: This test controls the Type I experimentwise error rate, but it generally has a higher Type II error rate than REGWQ.

Alpha	0.05
Error Degrees of Freedom	184
Error Mean Square	0.119664
Critical Value of t	3.17001
Minimum Significant Difference	0.3166

Means with the same letter are not significantly different.

Bon Grouping			Mean	N	SITE
	A		0.68417	24	E1
	A				
B	A		0.51542	24	E6
B	A				
B	A	C	0.45875	24	E8
B		C			
B	D	C	0.30375	24	E3
B	D	C			
B	D	C	0.26333	24	R3
	D	C			
	D	C	0.19333	24	E4
	D				
	D		0.12958	24	R1
	D				
	D		0.10000	24	R6

-- RESIDUE=DIA --

The ANOVA Procedure

Class Level Information

Class	Levels	Values
SITE	8	E1 E3 E4 E6 E8 R1 R3 R6

Number of observations 192

Dependent Variable: UG

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	7	1.21022500	0.17288929	4.14	0.0003
Error	184	7.67894167	0.04173338		
Corrected Total	191	8.88916667			

R-Square	Coeff Var	Root MSE	UG Mean
0.136146	75.54545	0.204287	0.270417

Source	DF	Anova SS	Mean Square	F Value	Pr > F
SITE	7	1.21022500	0.17288929	4.14	0.0003

Levene's Test for Homogeneity of UG Variance
ANOVA of Squared Deviations from Group Means

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
SITE	7	0.0781	0.0112	1.07	0.3813
Error	184	1.9095	0.0104		

Bartlett's Test for Homogeneity of UG Variance

Source	DF	Chi-Square	Pr > ChiSq
SITE	7	35.1037	<.0001

ANOVA FOR CHEMICAL RESIDUES ACROSS SAMPLING AREAS 78

-- RESIDUE=DIA --

The ANOVA Procedure

Bonferroni (Dunn) t Tests for UG

NOTE: This test controls the Type I experimentwise error rate, but it generally has a higher Type II error rate than REGWQ.

Alpha	0.05
Error Degrees of Freedom	184
Error Mean Square	0.041733
Critical Value of t	3.17001
Minimum Significant Difference	0.1869

Means with the same letter are not significantly different.

Bon Grouping		Mean	N	SITE
	A	0.40208	24	E8
	A			
	A	0.39042	24	E1
	A			
B	A	0.27875	24	E6
B	A			
B	A	0.25375	24	E3
B	A			
B	A	0.24000	24	R3
B	A			
B	A	0.23333	24	E4
B				
B		0.19958	24	R1
B				
B		0.16542	24	R6

ANOVA FOR CHEMICAL RESIDUES ACROSS SAMPLING AREAS 79

-- RESIDUE=TERB --

The ANOVA Procedure

Class Level Information

Class	Levels	Values
SITE	8	E1 E3 E4 E6 E8 R1 R3 R6

Number of observations 192

Dependent Variable: UG

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	7	118.3988167	16.9141167	18.58	<.0001
Error	184	167.4817500	0.9102269		
Corrected Total	191	285.8805667			

R-Square	Coeff Var	Root MSE	UG Mean
0.414155	79.14758	0.954058	1.205417

Source	DF	Anova SS	Mean Square	F Value	Pr > F
SITE	7	118.3988167	16.9141167	18.58	<.0001

Levene's Test for Homogeneity of UG Variance
ANOVA of Squared Deviations from Group Means

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
SITE	7	65.4317	9.3474	5.16	<.0001
Error	184	333.2	1.8111		

Bartlett's Test for Homogeneity of UG Variance

Source	DF	Chi-Square	Pr > ChiSq
SITE	7	41.4268	<.0001

-- RESIDUE=TERB --

The ANOVA Procedure

Bonferroni (Dunn) t Tests for UG

NOTE: This test controls the Type I experimentwise error rate, but it generally has a higher Type II error rate than REGWQ.

Alpha	0.05
Error Degrees of Freedom	184
Error Mean Square	0.910227
Critical Value of t	3.17001
Minimum Significant Difference	0.8731

Means with the same letter are not significantly different.

Bon Grouping			Mean	N	SITE
	A		2.8488	24	E1
	B		1.7792	24	E6
	B				
C	B		1.6154	24	E8
C	B				
C	B	D	1.0804	24	R6
C		D			
C		D	0.8767	24	E3
		D			
		D	0.5625	24	E4
		D			
		D	0.5571	24	R3
		D			
		D	0.3233	24	R1

Data Evaluation Report on Tissue Pesticide Residues and Histology of the Larynx and Gonads in Native Green Frogs (*Rana clamitans*) Collected from Agricultural Areas in Michigan: Hormone Analysis.

EPA MRID Number 458677-02

Data Requirement:

EPA DP Barcode	D288775	
EPA MRID		458677-02
EPA Guideline		70-1(Special Study)

Test material:

Purity: not reported

Common name Atrazine

Chemical name: IUPAC

CAS name 6-chloro-N-ethyl-N'-(1-methylethyl)-1,3,5-triazine-2,4-diamine

CAS No. 1912-24-9

Synonyms

EPA PC Code: 80803

Thomas M Steeger

Primary Reviewer: Thomas M. Steeger, Ph.D., Senior Biologist **Date:** March 27, 2003
Environmental Fate and Effects Division, ERB 4,
U. S. Environmental Protection Agency

Joseph E Tietge

Secondary Reviewer(s): Joseph E. Tietge, M.S., Research Aquatic Biologist **Date:** April 16, 2003
Mid-Continent Ecology Division, National Health and Environmental Effects Research Laboratory (Duluth), U. S. Environmental Protection Agency

Mary Frankenberry for Stephanie Irene, Ph.D., Senior Advisor **Date:** 4/28/03
Environmental Fate and Effects Division, ERB 3, U. S. Environmental Protection Agency

Mary Frankenberry Mary J. Frankenberry, Senior Statistician **Date:** 4/28/03
Environmental Fate and Effects Division, ERB 3, U. S. Environmental Protection Agency

EPA PC Code 080803

Date Evaluation Completed: 06/01/2003

<u>**CITATION**</u>: Jones, P. D., M. B. Murphy, M. Hecker, J. P. Giesy. 2003. Tissue Pesticide Residues and Histology of the Larynx and Gonads in Native Green Frogs (*Rana clamitans*) Collected from Agricultural Areas in Michigan: Hormone Analysis. Aquatic Toxicology Laboratory, Michigan State University, 218C National Food Safety and Toxicology Center, E. Lansing, MI. Sponsor: Syngenta Crop Protection, Inc. Laboratory Study ID: ECORISK Number MSU-02.

EXECUTIVE SUMMARY:

This represents a summary for Phase I of a three-year study where primarily the green frog (*Rana clamitans*) but also the leopard frog (*Rana pipiens*) and the bullfrog (*Rana catesbeiana*) were collected from three reference ponds and six atrazine-exposed ponds. Both juvenile (372) and adult (340) green frogs were collected and examined for gross gonadal abnormalities and blood plasma estradiol and testosterone levels. A total of four mixed or unknown sex animals were identified in all of the frogs collected. Hormone levels exhibited considerable variability among locations and individuals. No relationship between plasma hormone levels and atrazine exposure could be determined.

While this represents an interim report (Phase 1) of a three-year study, the results suggest that *Rana clamitans* are not markedly impacted by atrazine exposure in terms of gonadal deformities. However, reference sites contained atrazine so the ability of this field study to discriminate effects may be limited. Additionally, sex steroid levels exhibited considerable variability, and the study was not able to distinguish meaningful differences between "exposed" and "reference" sites. Plasma steroid levels are of questionable value because data were collected over a time period of four months for adult frogs, and coefficients of variability ranged as high as 10,628%. There were no gender-specific estradiol levels. Exposed males contained roughly similar (0.91) amounts of estradiol as females, while in reference sites males exhibited roughly 4.9 times the plasma estradiol concentration than females.

Because of the variability associated with plasma steroid hormone levels measured in this study, it is unlikely that the study will be able to differentiate any treatment-related effects, especially since the reference sites may have contained low levels of atrazine.

I. MATERIALS AND METHODS

GUIDELINE FOLLOWED: Nonguideline Study

COMPLIANCE: Not conducted under full GLP; however, most practices as defined by 40 CFR Part 160, August 19, 1989 were established for this study, including but not limited to:

- Written, authorized protocol
- Written, authorized Standard Operating Procedures for all key procedures.
- Organization and Personnel were sufficient in terms of number, education, training and experience.
- Facilities were of suitable size and construction
- Equipment used was of appropriate design and adequate capacity.
- Independent QA Inspection were conducted.
- Final Report was written
- Raw data, documentation, records, protocols, and final report were archived.

A. MATERIALS:

1. Test Material Atrazine

Description: Not reported

Lot No./Batch No. : Not reported

Purity: NA

Stability of compound under test conditions: Not reported

Storage conditions of test chemicals:_ Not reported

2. Test organism:

Species: Green Frogs (*Rana clamitans*)
Age at test initiation: Juveniles and adults
Weight at study initiation: (mean and range) Not reported
Length at study initiation: (mean and range) Not reported

Source: Field-collected at nine (3 reference and 6 atrazine-exposed) sites located in Livingston, Ingham, Barry and Lapeer counties, Michigan

B. STUDY DESIGN:

Objective: To assess the effects of atrazine on kidney and gonad histology and plasma steroid hormone concentrations and gonadal aromatase activity of green frogs (*R. clamitans*) and other incidental ranid species collected from various field sites in Michigan.

1. Experimental Conditions

Experimental sites (KZ02, KZ03, KZ07, LPR02, LPR03 and HW01) were based on proximity to agriculture where atrazine was used and where the genus *Rana* were located. Reference sites (HG01, HL01 and LPR08) were selected in non-agricultural areas.

During each sampling,water chemistry (conductivity, DO, pH and temperature at each of 4 points on water's surface) samples were collected,and on the first visit a sediment (5-cm grab) sample was collected. All samples were analyzed for atrazine using Envirogard triazine ELISA kit (LOD = 0.025 μg/L); sediment samples will be extracted and then analyzed later using ELISA. Results of ELISA will be verified using GC-MS.

Sampling (from 8 PM to midnight) was conducted three times throughout the Summer of 2002. Tadpoles were collected in un-baited minnow traps in the late spring and early summer; juvenile frogs were sampled in mid-summer (June and July); adult frogs were sampled at the end of summer (September). Frogs were sampled using fish nets and were held in buckets for less than two hours to minimize sampling stress and affects on plasma sex steroid concentrations.

Frogs were euthanized by immersion in MS-222, then weighed and measured (SVL) before blood collection via cardiac puncture; blood collection was completed within an approximate four-hour window of time.

Frogs were dissected and examined under a microscope for gonad morphology. One gonad was collected from adult and juvenile frogs, and snap frozen for use in aromatase assay. The other gonad was left in the carcass and fixed in Bouin's solution. Gonadal somatic index (partial GSI) was calculated based on the weight of single gonad.

Plasma samples were extracted with diethyl ether. The level of quantitation (LOQ) for testosterone and 17-β estradiol was 0.78 - 800 pg/well.

II. RESULTS and DISCUSSION: [All results discussed in this section and the next are those reported by the study authors. Although supplemental data are typically used in a qualitative manner only, EFED verified spreadsheet data and ran basic statistical analyses on the major study parameters. See attached appendix. If results differed in any substantive way, the difference was reported in the text below.]

Quantifiable levels of atrazine were detected in all (exposed and reference) of the ponds sampled. Atrazine concentrations in exposed ponds ranged from 0.025 to 250 µg/L. Reference pond water atrazine concentrations ranged from 0.015 to 0.093 µg/L. No data were reported on other triazine herbicides, degradates or other pesticide residues. Although the study notes that maximum concentrations did not appear to persist for very long at the sites, with measurements tending to drop off in the next water sample, intervals between sampling make it difficult to support this observation.

Table 1. Atrazine water concentrations in both exposed and reference collection sites during spring (May), early summer (June and July), mid-summer (August) and late summer (September) samples.

Site	Atrazine Water Concentrations (µg/L)			
	Spring	Early-Summer	Mid-Summer	Late-Summer
KZ02 (Exposed 1)	0.52	0.094	0.025	0.030
KZ03 (Exposed 2)	1.6	1.8	0.71	0.11
KZ07 (Exposed 3)	0.031	0.48	0.17	0.11
HW01 (Exposed 4)	0.12	0.36	0.24	0.24
LPR02 (Exposed 5)	0.83	6.5×10^1	2.9	0.078
LPR03 (Exposed 6)	2.5×10^2	0.69	0.092	1.9
HL01 (Reference 1)	0.058	0.069	0.040	0.015
HG01 (Reference 2)	0.026	0.093	0.069	0.067
LPR08 (Reference 3)	0.085	0.062	0.033	0.036

Of the 372 juvenile and 340 adult green frogs collected, four (0.6%) had mixed or unknown sex. It is assumed that this is based on dissecting scope observations because it states that animals will be subject to histopathology in upcoming months.

GSI varied considerably between sampling sites. Female GSI was greatest at HL01 reference site and at the HW01 and LPR02 exposed sites.

Within-site and between-site variability in testosterone (T) levels was high (**Tables 2 and 3**). Testosterone was highest at LPR08 reference site and KZ02 experimental site in both males and females

Estradiol (E_2) concentrations in both male and females was greatest at KZ02 exposed site and LPR08 reference site (**Tables 2 and 3**).

The ratio of plasma estradiol to testosterone levels (E/T) in females averaged 1.7 ± 1.6 at atrazine-exposed sites compared to 1.6 ± 1.0 at reference sites (**Table 4**). For males, E/T ratios averaged 1.28 ± 1.7 and 0.95 ± 0.6 for atrazine-exposed and reference sites, respectively.

Table 2. Mean plasma testosterone and estradiol in female green frogs (*Rana clamitans*) collected in reference and atrazine-exposed sites in Michigan. Standard deviations (Std. Dev.) and coefficients of variability (CV = [std. dev. ÷ mean] x 100).

Site	Female *Rana clamitans* Mean Testosterone pg/mL	Std. Dev.	CV	Mean Estradiol pg/mL	Std. Dev.	CV
Exposed 1 (KZ02)	3697	641	17%	16407	5718	35%
Exposed 2 (KZ03)	198	2101	1061%	495	4573	924%
Exposed 3 (KZ07)	249	1684	676%	289	478	165%
Exposed 4 (HW01)	640	534	83%	25	226	904%
Exposed 5 (LPR02)	341	843	247%	68	826	1215%
Exposed 6 (LPR03)	209	188	90%	321	467	145%
Reference 1 (HL01)	180	646	359%	134	183	137%
Reference 2 (HG01)	134	98	73%	51	172	337%
Reference 3 (LPR08)	3681	3452	94%	5253	4430	84%

Table 3. Mean plasma testosterone and estradiol in male green frogs (*Rana clamitans*) collected in reference and atrazine-exposed sites in Michigan. Standard deviations (Std. Dev.) and coefficients of variability (CV = [std. dev. ÷ mean] x 100).

Site	Male *Rana clamitans* Mean Testosterone pg/mL	Std. Dev.	CV	Mean Estradiol pg/mL	Std. Dev.	CV
Exposed 1 (KZ02)	3300	8574	260%	14308	7668	54%
Exposed 2 (KZ03)	118	888	753%	238	7678	3226%
Exposed 3 (KZ07)	129	3555	2756%	20	1199	5995%
Exposed 4 (HW01)	89	--	--	26	--	--

Exposed 5 (LPR02)	168	173	103%	2.7	133	4926%
Exposed 6 (LPR03)	826	--	--	732	--	--
Reference 1 (HL01)	149	610	409%	36	3826	10628%
Reference 2 (HG01)	189	1733	917%	234	2956	1263%
Reference 3 (LPR08)	3183	4171	131%	4314	3568	83%

Table 4. Female and male plasma estradiol to testosterone (E/T) ratios for green frogs (*Rana clamitans*) collected from atrazine-exposed and reference sites.

Site	Female E/T Ratio	Male E/T Ratio
Exposed 1 (KZ02)	4.4	4.34
Exposed 2 (KZ03)	2.5	2.02
Exposed 3 (KZ07)	1.2	0.16
Exposed 4 (HW01)	0.04	0.29
Exposed 5 (LPR02)	0.20	0.02
Exposed 6 (LPR03)	1.54	0.89
Reference 1 (HL01)	0.74	0.24
Reference 2 (HG01)	0.38	1.24
Reference 3 (LPR08)	1.43	1.36

REVIEWER'S COMMENTS:

Because of the large number of sampling sites and the logistics of collecting and processing samples at night, it is understandable why sampling had to be conducted over a protracted period of time. However, while many of the experimental sites were sampled between 7/23/02 and 7/29/02, sampling of reference sites extended from 7/31/02 through 8/13/02. Only one exposed site (LPR02) was sampled as late as 8/13/02. When adult frogs were collected in September, reference sites were sampled relatively early (9/6 - 9/16), while experimental areas were sampled later (9/13 - 9/30). It's not clear when each of the samples was collected; there are sampling dates reported in the appendix, but these are not consistent.

. The major hypothesis that was being evaluated in this study is that atrazine exposure results in abnormal kidney and gonad development and that atrazine causes changes in plasma estradiol and testosterone levels. Because of the design of the experiment and the uncontrolled environmental factors in a field study, this hypothesis could not be tested. Furthermore, presumptive effects on gonadal development must occur at the appropriate time in development.. In this study, atrazine measurements were taken throughout the summer, but they were apparently not taken during the larval period. As a result, it is uncertain whether or not exposure occurred at the appropriate time to elicit a response.

Because there is considerable variability in the plasma steroid levels, it is difficult to determine potential treatment effects based on these measurements. Plasma estradiol levels in atrazine-exposed females ranged from 25 to 16,407 pg/mL with coefficients of variability (CV) ranging from 35% to 1,215%; at reference sites female plasma estradiol ranged from 51 to 5,253 pg/mL with CV's ranging from 84 to 337%. Testosterone levels in females were equally variable with coefficients of variability ranging between 17 and 1,061%; mean plasma testosterone levels in females from both atrazine-exposed and reference sites ranged roughly from 134 to 3,697 pg/mL. For males, plasma testosterone levels in atrazine-exposed animals ranged from 89 to 3,300 pg/mL (CV range 103 - 2,756%), while males from reference sites ranged from 149 to 3,183 pg/mL (CV range 131 - 917%). Plasma estradiol levels in males at atrazine-exposed sites ranged from 2.7 to 14,308 pg/mL (CV range 54 - 5,995%), while reference site males ranged from 36 - 4314 pg/mL (CV range 83 - 10,628%). Iinteresting enough the highest plasma estradiol ranges were measured in males collected from atrazine-exposed sites. Unfortunately, the highest variability (as reflected by CVs ranging as high as 10,628%) was observed in reference sites for male estradiol levels. With such high levels of variability and with the presence of atrazine in the reference sites, the potential to differentiate treatment effects would be remote, unless extremely large sample sizes were used. Alternatively the researchers could use a shorter time period for collecting and analyzing samples to limit the likelihood that frogs would be at different stages of their breeding cycle.

Gonadal anomalies were only detected in 0.6% of the total juvenile and adult frogs sampled; however, it is unclear whether histopathology will significantly alter that percentage.

The median ratio of estradiol to testosterone exceeded 1.0 (range: 1.9 - 8.6) in males collected from reference sites; however, at half of the exposed sites the median ratio was less than 1.0 (range 0.26 - 0.89). The remaining exposed sites (3) had median ratios ranging from 4.9 to 6.8.

Reference sites all proved to contain measurable levels of atrazine and were in some cases higher than exposed site values. No data were reported on other triazines and/or degradates nor were data provided on what other chemicals may have been present in the exposed and reference study sites. Although water quality data were collected, no data were provided for analysis.

While this represents an interim report (Phase 1) of a three-year study, the results suggest that *Rana clamitans* were not markedly impacted by atrazine exposure in terms of gonadal deformities. However, reference sites contained atrazine so the ability of this field study to discriminate effects may be limited. Additionally, sex steroid levels exhibited considerable variability, and the study was not able to distinguish meaningful differences between "exposed" and "reference" sites.

The summary tables for plasma steroid levels are of little value because they summarize data collected over 4 months in adult frogs. There were no gender-specific steroid hormone levels, both males and females exhibited roughly similar average ratios from atrazine exposed sites, *i.e.*, 1.7 versus 1.3, and from reference sites, *i.e.*, 0.85 and 0.95, suggesting that this study couldn't distinguish plasma steroid differences between males and females or between reference and exposure sites if such differences existed.

Although the study was not conducted under full GLP, the report notes that most practices were included, one of which involved the writing of a final report. The current study report does not constitute a final report and therefore a Final Report was not written.

CONCLUSIONS:

Although this study provides preliminary data on the incidence of abnormal gonads and the levels of plasma steroid hormone in green frogs collected from both reference and atrazine-exposed sites, there is no conclusive evidence that frogs from the two sites are statistically different relative to these measurement endpoints. However, the gonadal data are based on gross morphology and the incidence of abnormalities may change once histology is completed. Additionally, given the high level of variability in steroid hormone levels, it is unlikely that this study could have differentiated between males and females or between reference and atrazine-exposed sites had a difference existed. With coefficients of variability approaching 11,000%, the number of animals required to detect a specified difference with any reasonable level of confidence would be high given the study's current design.

AVERAGE WEIGHT OF FROGS COLLECTED BY AREA AND SEX 279

Obs	AREA	SEX	_TYPE_	_FREQ_	MEAN	STD	CV
1	HG01		0	1	.	.	.
2	HG01	F	0	4	44.9828	11.6195	25.8310
3	HG01	M	0	24	40.7518	11.0699	27.1643
4	HL01	F	0	7	45.7543	4.4464	9.7179
5	HL01	M	0	18	40.1478	7.7085	19.2003
6	HL02	F	0	2	73.0950	13.7674	18.8349
7	HW01	F	0	4	42.0025	23.7297	56.4958
8	HW01	M	0	1	18.4600	.	.
9	KZ03	F	0	13	36.5923	14.0417	38.3734
10	KZ03	M	0	9	30.5756	10.3927	33.9903
11	KZ07	F	0	22	32.2627	6.3247	19.6037
12	KZ07	M	0	28	29.9182	6.1725	20.6312
13	KZ07	Unk.	0	1	30.7600	.	.
14	LPRO	F	0	23	31.5283	15.8872	50.3904
15	LPRO	M	0	24	28.4154	10.1072	35.5696

NONPARAMETRIC COMPARISON OF BODY WEIGHT ACROSS SAMPLING AREAS BY SEX 280

-- SEX=F --

The NPAR1WAY Procedure

Wilcoxon Scores (Rank Sums) for Variable WEIGHT
Classified by Variable AREA

AREA	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
HG01	4	222.0	152.0	42.410690	55.500000
HL01	7	423.0	266.0	54.905980	60.428571
HL02	2	146.0	76.0	30.408332	73.000000
HW01	4	166.0	152.0	42.410690	41.500000
KZ03	13	481.0	494.0	71.446950	37.000000
KZ07	22	721.0	836.0	85.934083	32.772727
LPRO	23	691.0	874.0	87.032561	30.043478

Kruskal-Wallis Test

Chi-Square	19.6115
DF	6
Pr > Chi-Square	0.0032

NONPARAMETRIC COMPARISON OF BODY WEIGHT ACROSS SAMPLING AREAS BY SEX 281

-- SEX=F --

The NPAR1WAY Procedure

Median Scores (Number of Points Above Median) for Variable WEIGHT
Classified by Variable AREA

AREA	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
HG01	4	4.0	1.973333	0.979433	1.000000
HL01	7	7.0	3.453333	1.267999	1.000000
HL02	2	2.0	0.986667	0.702250	1.000000
HW01	4	2.0	1.973333	0.979433	0.500000
KZ03	13	6.0	6.413333	1.649997	0.461538
KZ07	22	8.0	10.853333	1.984563	0.363636
LPRO	23	8.0	11.346667	2.009931	0.347826

Median One-Way Analysis

Chi-Square	16.6089
DF	6
Pr > Chi-Square	0.0108

NONPARAMETRIC COMPARISON OF BODY WEIGHT ACROSS SAMPLING AREAS BY SEX 282

-- SEX=M --

The NPAR1WAY Procedure

Wilcoxon Scores (Rank Sums) for Variable WEIGHT
Classified by Variable AREA

AREA	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
HG01	24	1717.0	1260.00	129.614814	71.541667
HL01	18	1299.0	945.00	116.382988	72.166667
HW01	1	8.0	52.50	30.020826	8.000000
KZ03	9	385.0	472.50	86.494219	42.777778
KZ07	28	1160.0	1470.00	136.455121	41.428571
LPR0	24	891.0	1260.00	129.614814	37.125000

Kruskal-Wallis Test

Chi-Square	30.3302
DF	5
Pr > Chi-Square	<.0001

Median Scores (Number of Points Above Median) for Variable WEIGHT
Classified by Variable AREA

AREA	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
HG01	24	18.0	12.00	2.158748	0.750000
HL01	18	15.0	9.00	1.938371	0.833333
HW01	1	0.0	0.50	0.500000	0.000000
KZ03	9	3.0	4.50	1.440570	0.333333
KZ07	28	9.0	14.00	2.272674	0.321429
LPR0	24	7.0	12.00	2.158748	0.291667

Median One-Way Analysis

Chi-Square	23.5098
DF	5
Pr > Chi-Square	0.0003

AVERAGE LENGTH OF FROGS COLLECTED BY AREA AND SEX 284

Obs	AREA	SEX	_TYPE_	_FREQ_	MEAN	STD	CV
1	HG01		0	1	.	.	.
2	HG01	F	0	4	7.77475	0.62670	8.0608
3	HG01	M	0	24	7.28233	1.61161	22.1304
4	HL01	F	0	7	7.69100	0.34031	4.4247
5	HL01	M	0	18	7.50867	0.51030	6.7962
6	HL02	F	0	2	8.94050	0.84782	9.4829
7	HW01	F	0	4	7.38650	1.13260	15.3334
8	HW01	M	0	1	5.87600	.	.
9	KZ03	F	0	13	7.32492	1.02200	13.9523
10	KZ03	M	0	9	6.96600	0.75002	10.7669
11	KZ07	F	0	22	6.98036	0.48803	6.9915
12	KZ07	M	0	28	6.86843	0.51202	7.4547
13	KZ07	Unk.	0	1	7.08900	.	.
14	LPRO	F	0	23	6.79417	1.23124	18.1220
15	LPRO	M	0	24	6.73617	0.81422	12.0874

NONPARAMETRIC COMPARISON OF BODY LENGHT (SVL) ACROSS SAMPLING AREAS BY SEX 285

--- SEX=F ---

The NPAR1WAY Procedure

Wilcoxon Scores (Rank Sums) for Variable SVL
Classified by Variable AREA

AREA	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
HG01	4	221.0	152.0	42.410690	55.250000
HL01	7	393.0	266.0	54.905980	56.142857
HL02	2	142.0	76.0	30.408332	71.000000
HW01	4	178.0	152.0	42.410690	44.500000
KZ03	13	483.0	494.0	71.446950	37.153846
KZ07	22	712.0	836.0	85.934083	32.363636
LPRO	23	721.0	874.0	87.032561	31.347826

Kruskal-Wallis Test

Chi-Square	15.9314
DF	6
Pr > Chi-Square	0.0141

Median Scores (Number of Points Above Median) for Variable SVL
Classified by Variable AREA

AREA	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
HG01	4	4.0	1.973333	0.979433	1.000000
HL01	7	7.0	3.453333	1.267999	1.000000
HL02	2	2.0	0.986667	0.702250	1.000000
HW01	4	3.0	1.973333	0.979433	0.750000
KZ03	13	3.0	6.413333	1.649997	0.230769
KZ07	22	9.0	10.853333	1.984563	0.409091
LPRO	23	9.0	11.346667	2.009931	0.391304

Median One-Way Analysis

Chi-Square	19.3126
DF	6
Pr > Chi-Square	0.0037

NONPARAMETRIC COMPARISON OF BODY LENGHT (SVL) ACROSS SAMPLING AREAS BY SEX 287

--- SEX=M ---

The NPAR1WAY Procedure

Wilcoxon Scores (Rank Sums) for Variable SVL
Classified by Variable AREA

AREA	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
HG01	24	1642.00	1260.00	129.614123	68.416667
HL01	18	1251.00	945.00	116.382368	69.500000
HW01	1	9.00	52.50	30.020666	9.000000
KZ03	9	416.00	472.50	86.493758	46.222222
KZ07	28	1176.50	1470.00	136.454393	42.017857
LPR0	24	965.50	1260.00	129.614123	40.229167

Average scores were used for ties.

Kruskal-Wallis Test

Chi-Square	22.2193
DF	5
Pr > Chi-Square	0.0005

Median Scores (Number of Points Above Median) for Variable SVL
Classified by Variable AREA

AREA	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
HG01	24	17.0	12.00	2.158748	0.708333
HL01	18	12.0	9.00	1.938371	0.666667
HW01	1	0.0	0.50	0.500000	0.000000
KZ03	9	4.0	4.50	1.440570	0.444444
KZ07	28	11.0	14.00	2.272674	0.392857
LPR0	24	8.0	12.00	2.158748	0.333333

Average scores were used for ties.

Median One-Way Analysis

Chi-Square	11.1222
DF	5
Pr > Chi-Square	0.0490

AVERAGE GONAD WEIGHT OF FROGS COLLECTED BY AREA AND SEX 289

Obs	AREA	SEX	_TYPE_	_FREQ_	MEAN	STD	CV
1	HG01		0	1	.	.	.
2	HG01	F	0	4	0.53175	0.28141	52.922
3	HG01	M	0	24	0.08608	0.02893	33.607
4	HL01	F	0	7	0.79743	0.27233	34.151
5	HL01	M	0	18	0.07022	0.02304	32.815
6	HL02	F	0	2	4.20200	2.90055	69.028
7	HW01	F	0	4	0.90725	1.05756	116.568
8	HW01	M	0	1	0.02600	.	.
9	KZ03	F	0	13	0.43562	0.48035	110.269
10	KZ03	M	0	9	0.06600	0.02666	40.401
11	KZ07	F	0	22	0.27882	0.14849	53.257
12	KZ07	M	0	28	0.06400	0.01933	30.205
13	KZ07	Unk.	0	1	.	.	.
14	LPR0	F	0	23	0.35174	0.40409	114.884
15	LPR0	M	0	24	0.07358	0.08499	115.502

NONPARAMETRIC COMPARISON OF GONAD WEIGHT ACROSS SAMPLING AREAS BY SEX 290

--- SEX=F ---

The NPAR1WAY Procedure

Wilcoxon Scores (Rank Sums) for Variable GONAD
Classified by Variable AREA

AREA	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
HG01	4	189.00	152.0	42.409786	47.250000
HL01	7	433.00	266.0	54.904809	61.857143
HL02	2	148.00	76.0	30.407683	74.000000
HW01	4	188.00	152.0	42.409786	47.000000
KZ03	13	482.50	494.0	71.445426	37.115385
KZ07	22	715.50	836.0	85.932250	32.522727
LPR0	23	694.00	874.0	87.030704	30.173913

Average scores were used for ties.

Kruskal-Wallis Test

Chi-Square	19.6246
DF	6
Pr > Chi-Square	0.0032

Median Scores (Number of Points Above Median) for Variable GONAD
Classified by Variable AREA

AREA	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
HG01	4	3.0	1.973333	0.979433	0.750000
HL01	7	7.0	3.453333	1.267999	1.000000
HL02	2	2.0	0.986667	0.702250	1.000000
HW01	4	2.0	1.973333	0.979433	0.500000
KZ03	13	5.0	6.413333	1.649997	0.384615
KZ07	22	9.0	10.853333	1.984563	0.409091
LPR0	23	9.0	11.346667	2.009931	0.391304

Average scores were used for ties.

Median One-Way Analysis

Chi-Square	12.3288
DF	6
Pr > Chi-Square	0.0550

NONPARAMETRIC COMPARISON OF GONAD WEIGHT ACROSS SAMPLING AREAS BY SEX 292

-- SEX=M --

The NPAR1WAY Procedure

Wilcoxon Scores (Rank Sums) for Variable GONAD
Classified by Variable AREA

AREA	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
HG01	24	1642.00	1248.0	128.153570	68.416667
HL01	18	983.50	936.0	115.121717	54.638889
HW01	1	6.00	52.0	29.724299	6.000000
KZ03	9	417.00	468.0	85.604525	46.333333
KZ07	27	1335.00	1404.0	133.321509	49.444444
LPR0	24	972.50	1248.0	128.153570	40.520833

Average scores were used for ties.

Kruskal-Wallis Test

Chi-Square	13.8281
DF	5
Pr > Chi-Square	0.0167

Median Scores (Number of Points Above Median) for Variable GONAD
Classified by Variable AREA

AREA	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
HG01	24	17.0	11.883495	2.155602	0.708333
HL01	18	10.0	8.912621	1.936400	0.555556
HW01	1	0.0	0.495146	0.499976	0.000000
KZ03	9	3.0	4.456311	1.439908	0.333333
KZ07	27	13.0	13.368932	2.242529	0.481481
LPR0	24	8.0	11.883495	2.155602	0.333333

Average scores were used for ties.

Median One-Way Analysis

Chi-Square	8.9955
DF	5
Pr > Chi-Square	0.1092

AVERAGE GONADOSOMATIC INDEX (GSI) OF FROGS COLLECTED BY AREA AND SEX 294

Obs	AREA	SEX	_TYPE_	_FREQ_	MEAN	STD	CV
1	HG01		0	1	.	.	.
2	HG01	F	0	4	0.011275	0.004655	41.286
3	HG01	M	0	24	0.002137	0.000476	22.280
4	HL01	F	0	7	0.017357	0.005367	30.924
5	HL01	M	0	18	0.001717	0.000333	19.397
6	HL02	F	0	2	0.054700	0.029416	53.776
7	HW01	F	0	4	0.016925	0.011787	69.640
8	HW01	M	0	1	0.001400	.	.
9	KZ03	F	0	13	0.009985	0.006135	61.443
10	KZ03	M	0	9	0.002156	0.000343	15.921
11	KZ07	F	0	22	0.008295	0.003194	38.508
12	KZ07	M	0	28	0.002178	0.000571	26.234
13	KZ07	Unk.	0	1	.	.	.
14	LPR0	F	0	23	0.009487	0.007301	76.955
15	LPR0	M	0	24	0.002421	0.002454	101.367

NONPARAMETRIC COMPARISON OF GONADOSOMATIC INDEX (GSI) ACROSS SAMPLING AREAS BY SEX 295

--- SEX=F ---

The NPAR1WAY Procedure

Wilcoxon Scores (Rank Sums) for Variable GSI
Classified by Variable AREA

AREA	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
HG01	4	177.50	152.0	42.403752	44.375000
HL01	7	421.50	266.0	54.896998	60.214286
HL02	2	149.00	76.0	30.403357	74.500000
HW01	4	207.50	152.0	42.403752	51.875000
KZ03	13	469.50	494.0	71.435261	36.115385
KZ07	22	698.50	836.0	85.920025	31.750000
LPR0	23	726.50	874.0	87.018323	31.586957

Average scores were used for ties.

Kruskal-Wallis Test

Chi-Square	18.7491
DF	6
Pr > Chi-Square	0.0046

Median Scores (Number of Points Above Median) for Variable GSI
Classified by Variable AREA

AREA	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
HG01	4	3.0	1.973333	0.979433	0.750000
HL01	7	7.0	3.453333	1.267999	1.000000
HL02	2	2.0	0.986667	0.702250	1.000000
HW01	4	2.0	1.973333	0.979433	0.500000
KZ03	13	5.0	6.413333	1.649997	0.384615
KZ07	22	9.0	10.853333	1.984563	0.409091
LPR0	23	9.0	11.346667	2.009931	0.391304

Average scores were used for ties.

Median One-Way Analysis

```
Chi-Square          12.3288
DF                        6
Pr > Chi-Square      0.0550
```

NONPARAMETRIC COMPARISON OF GONADOSOMATIC INDEX (GSI) ACROSS SAMPLING AREAS BY SEX 297

-- SEX=M --

The NPAR1WAY Procedure

Wilcoxon Scores (Rank Sums) for Variable GSI
Classified by Variable AREA

AREA	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
HG01	24	1407.50	1248.0	127.893490	58.645833
HL01	18	580.00	936.0	114.888084	32.222222
HW01	1	13.50	52.0	29.663976	13.500000
KZ03	9	541.50	468.0	85.430796	60.166667
KZ07	27	1675.50	1404.0	133.050941	62.055556
LPR0	24	1138.00	1248.0	127.893490	47.416667

Average scores were used for ties.

Kruskal-Wallis Test

Chi-Square	15.1001
DF	5
Pr > Chi-Square	0.0099

Median Scores (Number of Points Above Median) for Variable GSI
Classified by Variable AREA

AREA	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
HG01	24	14.0	11.883495	2.155602	0.583333
HL01	18	4.0	8.912621	1.936400	0.222222
HW01	1	0.0	0.495146	0.499976	0.000000
KZ03	9	6.0	4.456311	1.439908	0.666667
KZ07	27	17.0	13.368932	2.242529	0.629630
LPR0	24	10.0	11.883495	2.155602	0.416667

Average scores were used for ties.

Median One-Way Analysis

Chi-Square	10.5912
DF	5
Pr > Chi-Square	0.0601

AVERAGE TESTOSTERONE CONCENTRATIONS BY AREA AND SEX COLLECTED 299

Obs	AREA	SEX	_TYPE_	_FREQ_	MEAN	STD	CV	MIN	MAX
1	HG01	F	0	9	149.68	98.03	65.496	49.87	307.18
2	HG01	M	0	38	513.61	1733.07	337.427	38.27	10715.78
3	HL02	F	0	2	2840.59	1307.26	46.021	1916.22	3764.96
4	HL01	F	0	13	590.12	646.18	109.498	57.32	1970.54
5	HL01	M	0	30	338.30	610.43	180.440	29.82	3068.27
6	HW01	F	0	4	648.93	533.77	82.254	73.90	1241.24
7	HW01	M	0	1	89.13	.	.	89.13	89.13
8	KZ02	F	0	16	4448.80	2370.51	53.284	1799.48	8897.31
9	KZ02	M	0	14	3504.74	1232.96	35.180	1281.92	5833.79
10	KZ03	F	0	17	1213.58	2101.09	173.132	14.26	8278.41
11	KZ03	M	0	13	604.88	887.89	146.788	24.32	2777.84
12	KZ07	F	0	22	226.46	115.27	50.901	58.45	407.51
13	KZ07	M	0	28	120.06	78.48	65.366	42.10	267.42
14	KZ07	U	0	1	54.52	.	.	54.52	54.52
15	LPR0	F	0	26	3377.91	3499.32	103.594	82.50	10347.86
16	LPR0	M	0	36	4061.89	4180.15	102.911	8.35	13154.07

ANALYSIS OF VARIANCE FOR TESTOSTERONE CONCENTRATION BETWEEN SAMPLING AREAS BY SEX 300

--- SEX=F ---

The GLM Procedure

Class Level Information

Class	Levels	Values
AREA	8	HG01 HL02 HLO1 HW01 KZ02 KZ03 KZ07 LPRO

Number of observations 109

NOTE: Due to missing values, only 105 observations can be used in this analysis.

Dependent Variable: T

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	7	277040769.7	39577252.8	8.19	<.0001
Error	97	468931321.7	4834343.5		
Corrected Total	104	745972091.4			

R-Square	Coeff Var	Root MSE	T Mean
0.371382	114.8531	2198.714	1914.371

Source	DF	Type I SS	Mean Square	F Value	Pr > F
AREA	7	277040769.7	39577252.8	8.19	<.0001

Source	DF	Type III SS	Mean Square	F Value	Pr > F
AREA	7	277040769.7	39577252.8	8.19	<.0001

ANALYSIS OF VARIANCE FOR TESTOSTERONE CONCENTRATION BETWEEN SAMPLING AREAS BY SEX 302

--- SEX=M ---

The GLM Procedure

Class Level Information

Class	Levels	Values
AREA	7	HG01 HL01 HW01 KZ02 KZ03 KZ07 LPR0

Number of observations 160

NOTE: Due to missing values, only 150 observations can be used in this analysis.

Dependent Variable: T

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	6	416991901	69498650	13.08	<.0001
Error	143	759843162	5313589		
Corrected Total	149	1176835063			

R-Square	Coeff Var	Root MSE	T Mean
0.354333	147.3356	2305.122	1564.538

Source	DF	Type I SS	Mean Square	F Value	Pr > F
AREA	6	416991901.3	69498650.2	13.08	<.0001

Source	DF	Type III SS	Mean Square	F Value	Pr > F
AREA	6	416991901.3	69498650.2	13.08	<.0001

PROC UNIVARIATE OUTPUT FOR RESIDUALS FROM GLM PROCEDURE USING TESTOSTERONE DATA BY SEX 306

--- SEX=F ---

The UNIVARIATE Procedure
Variable: Resid

Moments

N	105	Sum Weights	105
Mean	0	Sum Observations	0
Std Deviation	2123.43001	Variance	4508955.02
Skewness	1.55804522	Kurtosis	3.17959841
Uncorrected SS	468931322	Corrected SS	468931322
Coeff Variation	.	Std Error Mean	207.22555

Basic Statistical Measures

Location		Variability	
Mean	0.000	Std Deviation	2123
Median	-100.921	Variance	4508955
Mode	.	Range	10360
		Interquartile Range	1365

Tests for Location: Mu0=0

Test	-Statistic-		-----p Value------	
Student's t	t	0	Pr > |t|	1.0000
Sign	M	-12.5	Pr >= |M|	0.0187
Signed Rank	S	-629	Pr >= |S|	0.0438

Tests for Normality

Test	--Statistic---		-----p Value------	
Shapiro-Wilk	W	0.833207	Pr < W	<0.0001
Kolmogorov-Smirnov	D	0.220141	Pr > D	<0.0100
Cramer-von Mises	W-Sq	1.093384	Pr > W-Sq	<0.0050
Anderson-Darling	A-Sq	5.919474	Pr > A-Sq	<0.0050

Quantiles (Definition 5)

Quantile	Estimate
100% Max	7064.836
99%	6969.954
95%	4998.822
90%	2711.678
75% Q3	227.102

```
------------------------------------------- SEX=F -------------------------------------------

                              The UNIVARIATE Procedure
                                  Variable:  Resid

                              Quantiles (Definition 5)

                              Quantile        Estimate

                              50% Median      -100.921
                              25% Q1         -1138.011
                              10%            -2306.981
                              5%             -3036.410
                              1%             -3281.251
                              0% Min         -3295.404

                                Extreme Observations

                      ------Lowest-----         -----Highest-----

                         Value       Obs            Value       Obs

                      -3295.40        84          5896.93       105
                      -3281.25        89          6351.64       108
                      -3251.36        91          6823.05       102
                      -3196.13        86          6969.95       103
                      -3142.45        87          7064.84        45

                                   Missing Values

                                          -----Percent Of-----
                     Missing                              Missing
                       Value       Count      All Obs        Obs

                         .            4         3.67      100.00


------------------------------------------- SEX=F -------------------------------------------

                              The UNIVARIATE Procedure
                                  Variable:  Resid

                  Stem Leaf                             #              Boxplot
                     7 01                               2                 *
                     6 8                                1                 *
                     6 4                                1                 *
                     5 9                                1                 *
                     5 0                                1                 *
                     4
                     4 14                               2                 0
                     3 9                                1                 0
                     3
                     2 78                               2                 0
                     2 0                                1                 |
                     1 9                                1                 |
                     1 44                               2                 |
                     0 556889                           6                 |
                     0 0000111112222233444             19              +--+--+
                    -0 444433221111111111100000        24              *-----*
                    -0 997655555                        9              |     |
                    -1 332222222111000                 15              +-----+
                    -1 776                              3                 |
                    -2 3220                             4                 |
                    -2 9655                             4                 |
```

```
   -3 333210                      6                0
       ----+----+----+----+----
  Multiply Stem.Leaf by 10**+3


PROC UNIVARIATE OUTPUT FOR RESIDUALS FROM GLM PROCEDURE USING TESTOSTERONE DATA BY SEX  309

-------------------------------------------- SEX=F --------------------------------------------

                            The UNIVARIATE Procedure
                                Variable:  Resid

                             Normal Probability Plot
      7250+                                                        *
          |                                                   *  *
          |                                                  *
          |                                                 *
          |                                                          ++
          |                                                *       ++
          |                                               *    +++
      3750+                                              *   ++
          |                                                 ++
          |                                              +*+
          |                                            ++*
          |                                          ++  *
          |                                       +++   *
          |                                     ++    **
       250+                                  +++******
          |                               *******
          |                            ****
          |                        *****+
          |                       **++
          |                     ***+
          |                   **++
     -3250+*   * * ***+
           +----+----+----+----+----+----+----+----+----+----+
                -2        -1         0        +1        +2
```

PROC UNIVARIATE OUTPUT FOR RESIDUALS FROM GLM PROCEDURE USING TESTOSTERONE DATA BY SEX 310

-- SEX=M --

The UNIVARIATE Procedure
Variable: Resid

Moments

N	150	Sum Weights	150
Mean	0	Sum Observations	0
Std Deviation	2258.2335	Variance	5099618.54
Skewness	2.16873075	Kurtosis	7.13432301
Uncorrected SS	759843162	Corrected SS	759843162
Coeff Variation	.	Std Error Mean	184.383993

Basic Statistical Measures

Location		Variability	
Mean	0.000	Std Deviation	2258
Median	-244.809	Variance	5099619
Mode	.	Range	14256
		Interquartile Range	458.79593

Tests for Location: Mu0=0

Test	-Statistic-		-----p Value------	
Student's t	t	0	Pr > |t|	1.0000
Sign	M	-39	Pr >= |M|	<.0001
Signed Rank	S	-2514.5	Pr >= |S|	<.0001

Tests for Normality

Test	--Statistic---		-----p Value------	
Shapiro-Wilk	W	0.70622	Pr < W	<0.0001
Kolmogorov-Smirnov	D	0.307319	Pr > D	<0.0100
Cramer-von Mises	W-Sq	3.515333	Pr > W-Sq	<0.0050
Anderson-Darling	A-Sq	16.93825	Pr > A-Sq	<0.0050

Quantiles (Definition 5)

Quantile	Estimate
100% Max	10202.1644
99%	9092.1757
95%	5619.6492
90%	1567.9838
75% Q3	-11.1053
50% Median	-244.8092
25% Q1	-469.9013
10%	-2330.2361
5%	-3256.9046
1%	-3995.9020
0% Min	-4053.5445

```
                         Extreme Observations

           ------Lowest-----            ------Highest-----

              Value      Obs               Value      Obs

           -4053.54      130             7257.12      144
           -3995.90      128             7952.52      141
           -3958.06      131             9020.27      159
           -3829.78      129             9092.18      145
           -3776.94      127            10202.16       37


                            Missing Values

                                      -----Percent Of-----
           Missing                                 Missing
             Value       Count        All Obs          Obs

               .            10          6.25       100.00


     PROC UNIVARIATE OUTPUT FOR RESIDUALS FROM GLM PROCEDURE USING TESTOSTERONE DATA BY SEX   312

-------------------------------------------- SEX=M --------------------------------------------

                        The UNIVARIATE Procedure
                            Variable:  Resid

                  Histogram                                #             Boxplot
  10500+*                                                  1                *
       .*                                                  2                *
       .
   7500+*                                                  2                *
       .*                                                  1                *
       .*                                                  2                *
   4500+
       .*                                                  2                *
       .**                                                 4                *
   1500+**                                                 4                0
       .*********                                         18                0
       .**********************************************    91             +-----+
  -1500+***                                                5                0
       .*****                                              9                *
       .****                                               8                *
  -4500+*                                                  1                *
        ----+----+----+----+----+----+----+----+--
        * may represent up to 2 counts


                       Normal Probability Plot
          10500+                                                   *
               |                                               * *
               |
           7500+                                             **
               |                                            *
               |                                          **     +++
           4500+                                            +++++
               |                                         +***+
               |                                     ++++***
           1500+                                 ++++   **
               |                             +++++ ******
               |                   ******************
          -1500+                 *** ++++
               |              ****+++
               |  * ******++
          -4500+*  +++++
                +----+----+----+----+----+----+----+----+----+----+
```

```
                      -2         -1         0         +1        +2
```

```
PROC UNIVARIATE OUTPUT FOR RESIDUALS FROM GLM PROCEDURE USING TESTOSTERONE DATA BY SEX  315

                                The UNIVARIATE Procedure
                                    Variable:  Resid

                                    Schematic Plots

        |
  10000 +                        *
        |
        |                        *
        |
   8000 +                        *
        |                        *
        |            *
        |            *           *
   6000 +            *           *
        |                        *
        |            *
        |            *
   4000 +            0           *
        |                        *
        |            0           *
        |            0           *
   2000 +            |           *
        |            |           *
        |            |           0
        |            |           0
      0 +         *--+--*     *--+--*     *--+--*
        |         |     |     +-----+
        |         +-----+        0
        |            |           0
  -2000 +            |           0
        |            |           *
        |            0           *
        |            0           *
  -4000 +                        *
         ------------+-----------+-----------+-----------
   SEX                  F           M           U
```

NONPARAMETRIC COMPARISON OF PLASMA TESTOSTERONE (T) CONCENTRATION ACROSS COLLECTION AREAS B 316

--- SEX=F ---

The NPAR1WAY Procedure

Wilcoxon Scores (Rank Sums) for Variable T
Classified by Variable AREA

AREA	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
HG01	9	213.0	477.0	87.361319	23.666667
HL02	2	159.0	106.0	42.657551	79.500000
HL01	13	529.0	689.0	102.784564	40.692308
HW01	4	178.0	212.0	59.738318	44.500000
KZ02	16	1391.0	848.0	112.154655	86.937500
KZ03	17	659.0	901.0	114.955064	38.764706
KZ07	18	625.0	954.0	117.613775	34.722222
LPR0	26	1811.0	1378.0	134.698429	69.653846

Kruskal-Wallis Test

Chi-Square	50.1393
DF	7
Pr > Chi-Square	<.0001

Median Scores (Number of Points Above Median) for Variable T
Classified by Variable AREA

AREA	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
HG01	9	0.0	4.457143	1.441088	0.000000
HL02	2	2.0	0.990476	0.703667	1.000000
HL01	13	5.0	6.438095	1.695506	0.384615
HW01	4	2.0	1.980952	0.985427	0.500000
KZ02	16	16.0	7.923810	1.850072	1.000000
KZ03	17	7.0	8.419048	1.896267	0.411765
KZ07	18	0.0	8.914286	1.940124	0.000000
LPR0	26	20.0	12.876190	2.221948	0.769231

Median One-Way Analysis

Chi-Square	53.2436
DF	7
Pr > Chi-Square	<.0001

NONPARAMETRIC COMPARISON OF PLASMA TESTOSTERONE (T) CONCENTRATION ACROSS COLLECTION AREAS B 318

--- SEX=M ---

The NPAR1WAY Procedure

Wilcoxon Scores (Rank Sums) for Variable T
Classified by Variable AREA

AREA	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
HG01	37	2283.0	2793.50	229.370697	61.702703
HL01	30	1665.0	2265.00	212.837967	55.500000
HW01	1	34.0	75.50	43.300308	34.000000
KZ02	14	1791.0	1057.00	154.785874	127.928571
KZ03	13	762.0	981.50	149.702761	58.615385
KZ07	19	734.0	1434.50	176.974339	38.631579
LPR0	36	4056.0	2718.00	227.248762	112.666667

Kruskal-Wallis Test

Chi-Square	73.3827
DF	6
Pr > Chi-Square	<.0001

Median Scores (Number of Points Above Median) for Variable T
Classified by Variable AREA

AREA	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
HG01	37	11.0	18.50	2.648604	0.297297
HL01	30	9.0	15.00	2.457696	0.300000
HW01	1	0.0	0.50	0.500000	0.000000
KZ02	14	14.0	7.00	1.787353	1.000000
KZ03	13	6.0	6.50	1.728657	0.461538
KZ07	19	3.0	9.50	2.043569	0.157895
LPR0	36	32.0	18.00	2.624101	0.888889

Median One-Way Analysis

Chi-Square	56.2530
DF	6
Pr > Chi-Square	<.0001

AVERAGE ESTRADIOL CONCENTRATIONS BY AREA AND SEX COLLECTED 320

Obs	AREA	SEX	_TYPE_	_FREQ_	MEAN	STD	CV	MIN	MAX
1	HG01	F	0	9	136.09	171.55	126.061	18.88	541.24
2	HG01	M	0	38	1350.88	2956.38	218.848	2.50	15789.18
3	HL01	F	0	13	239.70	182.96	76.328	26.92	497.18
4	HL01	M	0	30	916.63	3825.62	417.356	25.33	18036.73
5	HL02	F	0	2	805.78	541.62	67.217	422.80	1188.77
6	HW01	F	0	4	129.45	225.55	174.237	1.11	466.49
7	HW01	M	0	1	25.79	.	.	25.79	25.79
8	KZ03	F	0	17	3222.80	4573.00	141.895	4.42	16236.64
9	KZ03	M	0	13	4821.49	7678.18	159.249	52.64	21183.02
10	KZ07	F	0	22	463.71	478.15	103.113	10.39	1887.19
11	KZ07	M	0	28	582.12	1199.15	205.997	6.27	5610.75
12	KZ07	U	0	1	174.92	.	.	174.92	174.92
13	KZO2	F	0	16	10770.41	4310.62	40.023	2744.22	19133.06
14	KZO2	M	0	14	10490.20	2634.88	25.118	6449.99	16600.01
15	LPRO	F	0	26	4599.44	4687.58	101.916	1.21	16969.95
16	LPRO	M	0	36	4739.09	3926.58	82.855	1.10	13756.41

ANALYSIS OF VARIANCE FOR ESTRADIOL CONCENTRATION BETWEEN SAMPLING AREAS BY SEX 321

--- SEX=F ---

The GLM Procedure

Class Level Information

Class	Levels	Values
AREA	8	HG01 HL01 HL02 HW01 KZ03 KZ07 KZ02 LPR0

Number of observations 109

NOTE: Due to missing values, only 105 observations can be used in this analysis.
Dependent Variable: E2

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	7	1338518159	191216880	15.87	<.0001
Error	97	1168404398	12045406		
Corrected Total	104	2506922556			

R-Square	Coeff Var	Root MSE	E2 Mean
0.533929	100.5534	3470.649	3451.549

Source	DF	Type I SS	Mean Square	F Value	Pr > F
AREA	7	1338518159	191216880	15.87	<.0001

Source	DF	Type III SS	Mean Square	F Value	Pr > F
AREA	7	1338518159	191216880	15.87	<.0001

ANALYSIS OF VARIANCE FOR ESTRADIOL CONCENTRATION BETWEEN SAMPLING AREAS BY SEX 323

-- SEX=M --

The GLM Procedure

Class Level Information

Class	Levels	Values
AREA	7	HG01 HL01 HW01 KZ03 KZ07 KZO2 LPRO

Number of observations 160

NOTE: Due to missing values, only 148 observations can be used in this analysis.

Dependent Variable: E2

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	6	1292294626	215382438	15.34	<.0001
Error	141	1979233920	14037120		
Corrected Total	147	3271528546			

R-Square	Coeff Var	Root MSE	E2 Mean
0.395012	119.6745	3746.614	3130.671

Source	DF	Type I SS	Mean Square	F Value	Pr > F
AREA	6	1292294626	215382438	15.34	<.0001

Source	DF	Type III SS	Mean Square	F Value	Pr > F
AREA	6	1292294626	215382438	15.34	<.0001

PROC UNIVARIATE OUTPUT FOR RESIDUALS FROM GLM PROCEDURE USING ESTRADIOL DATA BY SEX 327

--- SEX=F ---

The UNIVARIATE Procedure
Variable: Resid

Moments

N	105	Sum Weights	105
Mean	0	Sum Observations	0
Std Deviation	3351.81409	Variance	11234657.7
Skewness	1.5010092	Kurtosis	4.28501184
Uncorrected SS	1168404398	Corrected SS	1168404398
Coeff Variation	.	Std Error Mean	327.103561

Basic Statistical Measures

Location		Variability	
Mean	0.000	Std Deviation	3352
Median	-123.888	Variance	11234658
Mode	.	Range	21040
		Interquartile Range	2367

Tests for Location: Mu0=0

Test	-Statistic-		-----p Value------	
Student's t	t	0	Pr > |t|	1.0000
Sign	M	-12.5	Pr >= |M|	0.0187
Signed Rank	S	-426	Pr >= |S|	0.1744

Tests for Normality

Test	--Statistic---		-----p Value------	
Shapiro-Wilk	W	0.860111	Pr < W	<0.0001
Kolmogorov-Smirnov	D	0.213219	Pr > D	<0.0100
Cramer-von Mises	W-Sq	0.890385	Pr > W-Sq	<0.0050
Anderson-Darling	A-Sq	4.5607	Pr > A-Sq	<0.0050

Quantiles (Definition 5)

Quantile	Estimate
100% Max	13013.841
99%	12370.510
95%	5876.148
90%	3773.767
75% Q3	486.776
50% Median	-123.888
25% Q1	-1880.503
10%	-3569.116
5%	-4370.211
1%	-4598.227
0% Min	-8026.193

```
                    Extreme Observations

        ------Lowest-----        ------Highest-----

           Value      Obs           Value      Obs

        -8026.19       68         6199.51      105
        -4598.23       89         8362.65       74
        -4598.05       88        11923.26      102
        -4531.09       90        12370.51      103
        -4436.59       77        13013.84       40

                       Missing Values

                                 -----Percent Of-----
        Missing                               Missing
          Value       Count       All Obs        Obs

            .             4          3.67      100.00
```

```
  PROC UNIVARIATE OUTPUT FOR RESIDUALS FROM GLM PROCEDURE USING ESTRADIOL DATA BY SEX     329

-------------------------------------------- SEX=F --------------------------------------------

                        The UNIVARIATE Procedure
                            Variable:  Resid

             Stem Leaf                                   #             Boxplot
               13 0                                      1                *
               12 4                                      1                *
               11 9                                      1                *
               10
                9
                8 4                                      1                *
                7
                6 2                                      1                0
                5 29                                     2                0
                4 356                                    3                0
                3 23558                                  5                |
                2 24                                     2                |
                1 01467                                  5                |
                0 111222233344455567                    18             +--+--+
               -0 9765544444433322221111111111111110    34             *-----*
               -1 97653                                  5             +-----+
               -2 88877741                               8                |
               -3 764422210                              9                |
               -4 66544422                               8                |
               -5
               -6
               -7
               -8 0                                      1                0
                  ----+----+----+----+----+----+----
              Multiply Stem.Leaf by 10**+3
```

```
PROC UNIVARIATE OUTPUT FOR RESIDUALS FROM GLM PROCEDURE USING ESTRADIOL DATA BY SEX     330

------------------------------------------- SEX=F --------------------------------------------

                                   The UNIVARIATE Procedure
                                       Variable:  Resid

                             Normal Probability Plot
          13500+                                                  *
               |                                               *
               |                                             *
               |
               |
               |                                           *     ++
               |                                             +++
               |                                          *+++
               |                                        **+
               |                                     +***
               |                                  ++***
           2500+                               +++ *
               |                            +++   **
               |                         +++******
               |                    *********
               |                  **+++
               |                ***+
               |            ****+
               |   * * *****++
               |        +++
               |     +++
               |  +++
          -8500+*+
                +----+----+----+----+----+----+----+----+----+----+
                    -2        -1         0        +1        +2
```

PROC UNIVARIATE OUTPUT FOR RESIDUALS FROM GLM PROCEDURE USING ESTRADIOL DATA BY SEX 331

--- SEX=M ---

The UNIVARIATE Procedure
Variable: Resid

Moments

N	148	Sum Weights	148
Mean	0	Sum Observations	0
Std Deviation	3669.35639	Variance	13464176.3
Skewness	2.47973855	Kurtosis	8.42158109
Uncorrected SS	1979233920	Corrected SS	1979233920
Coeff Variation	.	Std Error Mean	301.619237

Basic Statistical Measures

Location		Variability	
Mean	0.000	Std Deviation	3669
Median	-762.279	Variance	13464176
Mode	.	Range	21889
		Interquartile Range	1179

Tests for Location: Mu0=0

Test	-Statistic-		-----p Value------	
Student's t	t	0	Pr > |t|	1.0000
Sign	M	-38	Pr >= |M|	<.0001
Signed Rank	S	-1821	Pr >= |S|	0.0004

Tests for Normality

Test	--Statistic---		-----p Value------	
Shapiro-Wilk	W	0.727461	Pr < W	<0.0001
Kolmogorov-Smirnov	D	0.256757	Pr > D	<0.0100
Cramer-von Mises	W-Sq	2.560058	Pr > W-Sq	<0.0050
Anderson-Darling	A-Sq	12.75496	Pr > A-Sq	<0.0050

Quantiles (Definition 5)

Quantile	Estimate
100% Max	17120.0961
99%	16361.5293
95%	6152.1951
90%	3682.6693
75% Q3	-17.4236
50% Median	-762.2792
25% Q1	-1196.6524
10%	-4040.2126
5%	-4729.7052
1%	-4737.9875
0% Min	-4768.8486

```
                         Extreme Observations

              ------Lowest-----         ------Highest-----

                 Value      Obs              Value      Obs

              -4768.85       70            9017.32      156
              -4737.99      126           14438.29       37
              -4737.71      128           15862.95       80
              -4736.44      130           16361.53       79
              -4736.35      129           17120.10       47


                            Missing Values

                                      -----Percent Of-----
              Missing                              Missing
                Value        Count      All Obs        Obs

                  .             12         7.50     100.00
```

```
PROC UNIVARIATE OUTPUT FOR RESIDUALS FROM GLM PROCEDURE USING ESTRADIOL DATA BY SEX     333

------------------------------------------ SEX=M ------------------------------------------

                         The UNIVARIATE Procedure
                            Variable:  Resid

                     Histogram                         #             Boxplot
         17500+*                                       1                *
              .*                                       1                *
              .*                                       1                *
              .*                                       1                *
              .
              .
              .
              .
              .*                                       1                *
              .*                                       1                *
              .*                                       1                *
          6500+**                                      3                *
              .*                                       2                *
              .*                                       2                *
              .**                                      4                0
              .****                                    8                0
              .*                                       2                0
              .*****                                   9                +
              .***************************            54             +-----+
              .******************                     35             +-----+
              .***                                     5                |
              .*                                       1                0
         -4500+********                               16                0
               ----+----+----+----+----+--
               * may represent up to 2 counts
```

```
      PROC UNIVARIATE OUTPUT FOR RESIDUALS FROM GLM PROCEDURE USING ESTRADIOL DATA BY SEX     334

-------------------------------------------- SEX=M --------------------------------------------

                                  The UNIVARIATE Procedure
                                      Variable:  Resid

                                   Normal Probability Plot
                  17500+                                                     *
                       |                                                   *
                       |                                                 *
                       |                                                *
                       |
                       |
                       |
                       |
                       |
                       |                                               *    +
                       |                                              *  +++
                       |                                             *  ++
                   6500+                                           **+++
                       |                                          **+
                       |                                        ++**
                       |                                      ++ **
                       |                                   +++***
                       |                                +++   *
                       |                             +++    ***
                       |                          ***********
                       |                   ********
                       |                 **  +++
                       |                 * ++
                  -4500+* * *********++
                        +----+----+----+----+----+----+----+----+----+----+
                            -2        -1         0        +1        +2

                         |            0           *
                    5000 +            0           *
                         |            0
                         |            |           *
                         |            |           0
                    2500 +            |           0
                         |            |           0
                         |            |           |
                         |         +------+       |
                       0 +         *--+--*     +--+--+      *--+--*
                         |         |      |    *-----*
                         |         |      |    +-----+
                         |         +------+       |
                   -2500 +            |           |
                         |            |           0
                         |            |           0
                         |            |           0
                   -5000 +                        0
                         |
                         |
                         |
                   -7500 +
                         |            0
                         |
                         |
                  -10000 +
                          ------------+-----------+-----------+-----------
                     SEX              F           M           U
```

NONPARAMETRIC COMPARISON OF PLASMA ESTRADIOL CONCENTRATION ACROSS COLLECTION AREAS BY SEX 338

--- SEX=F ---

The NPAR1WAY Procedure

Wilcoxon Scores (Rank Sums) for Variable E2
Classified by Variable AREA

AREA	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
HG01	9	202.0	477.0	87.361319	22.444444
HL01	9	296.0	477.0	87.361319	32.888889
HL02	2	109.0	106.0	42.657551	54.500000
HW01	4	71.0	212.0	59.738318	17.750000
KZ03	17	910.0	901.0	114.955064	53.529412
KZ07	22	838.0	1166.0	127.002625	38.090909
KZO2	16	1467.0	848.0	112.154655	91.687500
LPR0	26	1672.0	1378.0	134.698429	64.307692

Kruskal-Wallis Test

Chi-Square	53.0292
DF	7
Pr > Chi-Square	<.0001

Median Scores (Number of Points Above Median) for Variable E2
Classified by Variable AREA

AREA	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
HG01	9	0.0	4.457143	1.441088	0.000000
HL01	9	0.0	4.457143	1.441088	0.000000
HL02	2	1.0	0.990476	0.703667	0.500000
HW01	4	0.0	1.980952	0.985427	0.000000
KZ03	17	8.0	8.419048	1.896267	0.470588
KZ07	22	8.0	10.895238	2.095000	0.363636
KZO2	16	16.0	7.923810	1.850072	1.000000
LPR0	26	19.0	12.876190	2.221948	0.730769

Median One-Way Analysis

Chi-Square	44.7975
DF	7
Pr > Chi-Square	<.0001

NONPARAMETRIC COMPARISON OF PLASMA ESTRADIOL CONCENTRATION ACROSS COLLECTION AREAS BY SEX 340

--- SEX=M ---

The NPAR1WAY Procedure

Wilcoxon Scores (Rank Sums) for Variable E2
Classified by Variable AREA

AREA	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
HG01	35	2434.0	2607.50	221.603061	69.542857
HL01	22	1021.0	1639.00	185.523583	46.409091
HW01	1	23.0	74.50	42.722945	23.000000
KZ03	13	1070.0	968.50	147.618596	82.307692
KZ07	27	1160.0	2011.50	201.408168	42.962963
KZO2	14	1856.0	1043.00	152.622628	132.571429
LPRO	36	3462.0	2682.00	223.749860	96.166667

Kruskal-Wallis Test

Chi-Square	61.2901
DF	6
Pr > Chi-Square	<.0001

Median Scores (Number of Points Above Median) for Variable E2
Classified by Variable AREA

AREA	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
HG01	35	11.0	17.50	2.593490	0.314286
HL01	22	3.0	11.00	2.171241	0.136364
HW01	1	0.0	0.50	0.500000	0.000000
KZ03	13	6.0	6.50	1.727627	0.461538
KZ07	27	9.0	13.50	2.357143	0.333333
KZO2	14	14.0	7.00	1.786190	1.000000
LPRO	36	31.0	18.00	2.618615	0.861111

Median One-Way Analysis

Chi-Square	52.9594
DF	6
Pr > Chi-Square	<.0001

Data Evaluation Report on a Pilot Study of Larval *R. clamitans* Response to Atrazine Exposure in Terms of Metamorphosis, Gonadal and Laryngeal Morphology and Selected Hormonal and Enzymatic Activities.
EPA MRID Number 458677-03

Data Requirement::

EPA DP Barcode	D288775
EPA MRID	458677-03
EPA Guideline	70-1(Special Study)

Test material: **Purity:** 97.1%

Common name Atrazine
Chemical name: IUPAC
CAS name 6-chloro-N-ethyl-N'-(1-methylethyl)-1,3,5-triazine-2,4-diamine
CAS No. 1912-24-9
Synonyms
EPA PC Code: 80803

Thomas M Steeger

Primary Reviewer: Thomas M. Steeger, Ph.D., Senior Biologist **Date:** March 27, 2003
Environmental Fate and Effects Division, ERB 4,
U. S. Environmental Protection Agency

Joseph E Tietge

Secondary Reviewer(s): Joseph E. Tietge, M.S., Research Aquatic Biologist **Date:** April 16, 2003
Mid-Continent Ecology Division, National Health and Environmental Effects Research Laboratory (Duluth),
U. S. Environmental Protection Agency

Stephanie R. Irene

Stephanie Irene, Ph.D., Senior Advisor **Date:** 4/29/03
Environmental Fate and Effects Division, ERB 3, U. S. Environmental Protection Agency

Mary Jan Frankenberry

Mary J. Frankenberry, Senior Statistician **Date:** 4/28/03
Environmental Fate and Effects Division, ERB 3, U. S. Environmental Protection Agency

EPA PC Code 080803

Date Evaluation Completed: 05/31/2003

CITATION: Hecker, M., K. K. Coady, D. L. Villeneuve, M. B. Murphy, P. D. Jones and J. P. Giesy. 2003. A Pilot Study of Response of Larval *Rana clamitans* to Atrazine Exposure: Assessment of Metamorphosis and Gonadal and Laryngeal Morphology and Selected Hormones and Enzyme Activities. Aquatic Toxicology Laboratory, Michigan State University, National Food Safety and Toxicology Center, E. Lansing, MI. Sponsor: Syngenta Crop Protection, Inc., Laboratory Study ID ECORISK Number MSU-03.

EXECUTIVE SUMMARY:

Green frog (*Rana clamitans*) tadpoles reared from field-collected eggs were exposed for 273 days, beginning 5-days post-hatch, to two concentrations (10 and 25 µg/L) of atrazine. Positive controls, dihydroxytestosterone and 17-β estradiol (0.1 µg/mL in 0.005% ethanol), a negative control (water) and a solvent control (0.005% ethanol) were also run. Replicates (9) consisted of 30 free-swimming tadpoles each. Initially,(exposure days 0 - 67) animals were maintained under static renewal conditions in 4 L of test solution; 50% tank changes were conducted every 72 hours. From Days 68 to Day 273, tadpoles were maintained in tanks containing 16L of test solution under static renewal. After 273 days, exposures were terminated and tadpoles were maintained in continuous flow-through 10-L glass tanks housed in large acrylic tanks containing 80 L of continuously renewing freshwater. At metamorphosis (fore-limb emergence), tadpoles were either housed individually or in small groups in 10-L glass tanks containing approximately 500 mL of freshwater. Over the study period, mortality across all treatment groups averaged 76.5% and was attributed to poor water quality and overcrowding during the 273- day static-renewal phase of the study. While mean-measured concentrations of atrazine were relatively consistent with nominal values, measurements were made on freshly prepared stock solutions; hence it is unclear what atrazine concentrations were present in aged exposure solutions. Additionally, measurable levels of atrazine were detected in the negative controls. Although the concentrations of positive control hormones were not measured, the positive controls using dihyrdotestosterone and 17-β estradiol suggested that green frogs only reacted to androgenic chemicals resulting in predominately (97.6%) male frogs, while the frogs were not affected by estradiol. It is uncertain whether this means that green frogs are unresponsive to estrogenic chemicals, or whether there was sufficient estradiol in solution to elicit an effect. While no intersex (testicular and ovarian tissue in the same animal) was observed in any of the treatment groups, this observation was based on gross morphology, and apparently there were difficulties in discerning the presence of gonads using this process. While time to and age at metamorphosis and the size of metamorphs were reduced in frogs treated with 10 µg/L atrazine, there was no difference in these same parameters for frogs treated with 25 µg/L atrazine relative to negative controls. Although there were no dose dependent effects in green frogs related to atrazine treatment, only two concentrations were monitored. Additionally, because only a limited number of frogs survived to complete metamorphosis, the conclusions regarding sex ratio data are questionable.

No analysis of gonad histology is provided and no measurements were made of aromatase levels. Contrary to the GLP statement, this study represents an interim report and not a final report. .

The high mortality indicative of poor water quality and overcrowding and the lack of response to the positive estradiol control made it difficult for the study authors to test the hypothesis that atrazine exposure was associated with developmental effects in amphibians. The study did provide the authors with a better appreciation for the conditions under which green frogs should be housed, and it suggests that the green frog may not be adequate for examining the effects of atrazine on amphibian development..

I. MATERIALS AND METHODS

GUIDELINE FOLLOWED: Nonguideline Study
COMPLIANCE: Not conducted under full GLP; however, most practices as defined by 40 CFR Part 160, August 19, 1989 were established for this study, including but not limited to:

- Written, authorized protocol
- Written, authorized Stanadard Operating Procedures for all key procedures.
- Organization and Personnel were sufficient in terms of number, education, training and experience.
- Facilities were of suitable size and construction
- Equipment used was of appropriate design and adequate capacity.
- Independent QA Inspections were conducted.
- Final Report was written
- Raw data, documentation, records, protocols, and final report was archieved.

A. MATERIALS:

1. Test Material Atrazine

Description: Not reported

Lot No./Batch No. : Not reported

Purity: 97.1%
Stability of compound under test conditions: Not reported

Storage conditions of test chemicals: Not reported

2. Test organism:

Species: Green frog (*Rana clamitans*)

Age at test initiation: Larvae (Gosner Stage 25; approximately 5 days post-hatch
Weight at study initiation: (mean and range)
Length at study initiation: (mean and range)

Source: Eggs field-collected as a single mass of fertilized eggs from Giesy pond in Williamston, MI (7/10/2001)

B. STUDY DESIGN:

Objective: 1) To develop and validate methods of husbandry and exposure for conducting laboratory studies with *R. clamitans*.

2) To determine the response of larval *R. clamitans* to atrazine by assessing metamorphosis and reproduction indices when animals are exposed during larval development. Indices to be evaluated include:

- % initiating metamorphosis
- % completing metamorphosis
- time to metamorphosis
- fresh post-mortem body weight and snout-vent length
- incidence of gross gonadal abnormalities
- histology of the gonads.

1. Experimental Conditions

a) Range-finding Study: Current study represents a pilot study

b. Definitive Study

Table 1 . Experimental Parameters

Parameter	Details
Acclimation: period: Conditions: (same as test or not) Feeding: Health: (any mortality observed)	 8 days transitioned from pond to laboratory water over unspecified time period not reported
Duration of the test	506-day study of which 273 days exposed to test solutions
Test condition static/flow- through Type of dilution systemfor flow-through method. Renewal rate for static renewal	 static renewal NA 50% test solution change every 72 hours
Aeration, if any	NA

Parameter	Details
Test vessel	
Material: (glass/stainless steel)	glass
Size:	10 L
Fill volume:	4 L After 67 days of exposure, tadpoles transferred from 10L tanks to tanks containing 16 L of test solution. After 273 days, tadpoles transferred to continuous flow-through system of clean freshwater through a 10-L glass tank housed in large acrylic tanks containing 80 L of continuously renewing freshwater; once animals initiated metamorphosis (fore-limb emergence), removed from flow-through system and housed as individuals or small groups in 10-L glass tanks containing approximately 500 ml of freshwater.
Source of dilution water Quality:	Treated well water (MSU-University Research Containment Facility
Water parameters:	
Hardness	426 mg/L as $CaCO_3$ (static);
pH	7.87 (static);
Dissolved oxygen	8.0 mg/L (static); 6.1 mg/L (flow-through)
Total organic carbon	
Particulate matter	
Ammonia	0.04 mg/L (static); 0.02 mg/L (flow-through) (see reviewer's comments)
Nitrite	0.22 mg/L (static); 0.02 mg/L (flow-through)
Metals	
Pesticides	
Chlorine	
Temperature	21.3°C (static); 24.8°C (flow-through)
{Salinity for marine or estuarine species}	
Intervals of water quality measurement	NA

Parameter	Details
Number of replicates/groups: negative control: water solvent control: 0.005% ethanol treated ones: atrazine at 10 and 25 µg/L positive controls: dihydroxytestosterone and estradiol	 9 9 9 + 9 9 + 9
Number of organisms per replicate /groups: control: solvent control: treated ones:	 (30 tadpoles /rep) x 9 reps = 270 tadpoles 30 tadpoles /rep) x 9 reps = 270 tadpoles 30 tadpoles /rep) x 9 reps = 270 tadpoles
Biomass loading rate	30 tadpoles/4 L → 30 tadpoles/10 L
Test concentrations: nominal: measured:	 10 and 25 µg/L 13.8 and 28.1 µg a.i./L
Solvent (type, percentage, if used)	freshwater for atrazine; 0.005% ethanol for positive hormone controls
Lighting	not reported
Feeding	Appendix reports that frog brittle was analyzed yielding inconclusive results. Feeding regime is not reported
Recovery of chemical Level of Quantitation Level of Detection	ELISA (Envirogard Triazine®; Strategic Diagnostics Newark, DE)/Beacon Analytical triazine plate (Beacon Analytical Systems, Portland, ME) LOD 0.025 µg/L (Envirogard); 0.05 µg/L (Beacon)
Positive control {if used, indicate the chemical and concentrations}	dihydroxytestosterone 0.1 µg/L 17-β estradiol 0.1 µg/L both hormones in 0.005% ethanol
Other parameters, if any	NA

2. Observations:

Table 2: Observations

Criteria	**Details**
Parameters measured including the sublethal effects/toxicity symptoms	mortality; time to metamorphosis, number completing metamorphosis, age (days) at metamorphosis, length, weight, gonadal abnormalities, sex
Observation intervals	daily
Were raw data included?	
Other observations, if any	

Animals not reaching metamorphosis by 506 days were sacrificed.
All frogs completing metamorphosis were analyzed for gross morphology and histology of the gonads (no mention of kidneys).

II. RESULTS and DISCUSSION: [All results discussed in this section and the next are those reported by the study authors. Although supplemental data are typically used in a qualitative manner only, EFED verified spreadsheet data and ran basic statistical analyses on the major study parameters. See attached appendix. If results differed in any substantive way, the difference was reported in the text below.]

Exposures were initiated at 5 days post-hatch when tadpoles were free swimming and external gills were resorbed (Gosner stage 25). The feeding regime for the tanks was not discussed

Atrazine levels [of stock solutions] were measured following static renewal and therefore represented fresh as opposed to aged exposure solution values. Measurements were made using two different ELISA kits and yielded roughly similar exposure estimates (Table 3) over the course of the study. In general, mean-measured concentrations ranged from 112% to 159% of nominal. Atrazine was detected in the controls at concentrations that were within the LOQ (0.025) for the assay. Because data was not reported for the solvent control or either of the positive controls, it is not clear whether the contamination was limited to negative controls or across all treatments. Triazine ELISA kits did not arrive within the first 60 days of exposure, and it is unclear whether Syngenta was verifying exposure at this time or whether the study was based strictly on nominal concentrations during the first 60 days. Dead tadpoles were partially degraded, partially eaten and/or covered in fungus when discovered dead and therefore many of them could not be salvaged for later analysis. These results suggest poor tank conditions for supporting such rapid deterioration of the tadpoles.

No measurements were recorded for dihydroxytestosterone or estradiol in the positive controls.

Table 3. Nominal versus mean-measured atrazine concentrations.

Treatment	Atrazine (nominal) µg/L	Syngenta mean-measured µg/L	MSU mean-measured µg/L
Control	0	0.14 (0.07 - 0.23)	0.10 (0.06 - 0.17)
10 µg/L	10	15.91 (12.03 - 19.90)	11.76 (10.21 - 13.65)
25 µg/L	25	27.95 (24.92 - 31.24)	28.23 (25.14 _ 31.60)

Across all treatment groups, mortality averaged 76.5% (**Table 4**). Mortality was reported to be greatest during the first month of the exposure period and decreased as tadpoles grew older. According to the report, "mass mortality events occurred early in the study and usually occurred within a time span of 24 hours." Mortality rates declined after 273 days when tadpoles were transferred out of static renewal into flow-through water system. Although there was no significant difference in mortality between atrazine-treated and negative controls, there was a difference between the positive control treatments and the ethanol solvent control; the dihydroxytestosterone group had significantly fewer deaths. High mortality rates were potentially attributed to high ammonia levels in the static renewal systems.

By exposure Day 58, ammonia (NH_3) concentrations were between 0.8 - 0.9 mg/L and nitrite (NO_2) concentrations were as high as 3.0 mg/L

Because of the loss of so many animals, hormone concentrations were not analyzed as an endpoint in this study.

Table 4 . Average percent mortality for each treatment group over 506 day study period.

Treatment	Average % Mortality
Untreated Controls	79.2
Ethanol Control	74.8
Dihydroxytestosterone	62.7
17-β estradiol	85.7
10 µg/L	73.1
20 µg/L	83.3

The first initiation of metamorphosis was observed on exposure day 99 and the first completion of metamorphosis was observed on day 112. As of day 143, 10 tadpoles had completed metamorphosis. Between day 143 (December 7) and 285 (April 28), no tadpoles had initiated metamorphosis. Age at initiation and completion of metamorphosis was significantly different among the atrazine-treated groups and the untreated controls; frogs treated with 10 µg/L atrazine initiated and completed metamorphosis at a significantly older age compared to both untreated control frogs and frogs exposed to 25 µg/L (**Table 5**). Frogs in the estradiol treatment initiated metamorphosis at a significantly younger age as compared with both the ethanol control and frogs exposed to DHT.

Frogs treated with 10 µg/L atrazine were significantly shorter (SVL) than frogs in the 25 µg/L atrazine exposure group; however, there were no significant differences in weight between any of the treatment groups at metamorphic completion (**Table 6**).

The incidence of gross gonadal deformities ranged from 0 to 5.9% across all treatments with size incongruity between gonad pairs as the most commonly observed anomally. No intersex gonads (testicular and ovarian tissue within the same individual) were observed during gross inspections. In two frogs, gonad or gonad pairs could not be located in both the estradiol and DHT treatments.

Sex ratios in the atrazine and untreated controls did not differ significantly from the expected 50:50 male:female ratio (**Table 7**). While estradiol and ethanol control sex ratios did not differ from a ratio of 50:50, the DHT treated animals were 97.7% male.

Table 5. Number of green frogs surviving to and completing metamorphosis.

Treatment	Initial N	# Frogs Initiating Metamorphosis	# Frogs Completing Metamorphosis
Untreated Controls	285	58	44
Ethanol Controls	280	69	47
Dihydroxytestosterone	291	104	75
17-β estradiol	282	40	33
10 μg atrazine/L	292	77	64
25 μg atrazine/L	292	48	37

Table 6. Mean ages (days), lengths (cm), and weights (g) at metamorphosis for *R. clamitans*.

Treatment	Mean age at initiation	Mean age at completion	Mean Weight (g)	Mean Length (cm)
Untreated Controls	328.14	336.75	1.76	2.52
Ethanol Controls	349.99	359.68	1.56	2.47
Dihydroxytestosterone	350.48	368.53	1.50	2.42
17-β estradiol	329.73	342.15	1.64	2.57
10 μg atrazine/L	361.81	376.70	1.48	2.39
25 μg atrazine/L	335.27	342.14	1.64	2.54

Table 7. Percent male and female green frogs in each treatment.

Treatment	% Males	% Females
Untreated Controls	43.1	56.9
Ethanol Controls	47.4	50.9
Dihydroxytestosterone	97.7	2.3
17-β estradiol	36.8	63.2
10 μg atrazine/L	40.3	59.7
25 μg atrazine/L	40.9	59.1

C. REPORTED STATISTICS: Kolmogrov-Smirnov's One Sample test with Lillifor's transformation was used to assess whether or not the data sets were normally distributed. When normally distributed, ANOVA followed by Fisher's LSD was used to detect significant differences between treatment groups. For non-normally distributed data, non-parametric Kruskal-Wallis test/Mann-Whitney U Test was used. The Chi-square test was used to detect differences in expected sex ratios and Pearson's Chi-square was used to test for differences in the incidences of gross gonadal abnormalities.

D. VERIFICATION OF STATISTICAL RESULTS: Statistical analyses run using SAS® (Statistical Analysis System, Release 8.01, Cary, North Carolina); see attached output.

E. STUDY DEFICIENCIES: The feeding regime was not reported; however, the animals were apparently fed frog brittle. The appendix reports that a previous analysis of the food was "inconclusive". It is unclear what "inconclusive" refers to; however, an analysis of the food supply was apparently not run

Atrazine was detected in the negative control.

Water quality during the static renewal phase of the study was poor.

F. REVIEWER'S COMMENTS:

Although the study was not conducted under full GLP, the report notes that most practices were included, one of which involved writing a final report. The current study report does not constitute a final report and therefore a Final Report was not written.

A major problem in this study is the low survival rate which ranged from 37-14%. Although the report correctly notes that the rate of mortality decreased after the first 30 days, it was still substantial. For example, control mortality (as estimated from Figure 1) at 30 days was about 80 individuals. Mortality in the controls for the remainder of the test was about 120 individuals. This high mortality rate indicates severely inadequate methods and suggests that the study may not be useful..

The high mortality rates across all treatments coupled with data showing high ammonia/nitrite levels suggest that this study was probably compromised by poor water quality caused by overcrowding in a static renewal system. Because only 50% of the water was changed every 72 hours for the first 67 days of exposure, there is a high potential for waste products to accumulate.. The authors acknowledged that high mortality was probably caused by tadpole overcrowding in static tanks and that poor water quality (high ammonia and nitrite) may have contributed to mortality. The authors also acknowledged that these factors may have delayed growth and development of tadpoles because increased rates of development coincided with a shift from static to flow-through exposure systems. Tadpoles that underwent metamorphosis early tended to come from tanks that had experienced high mortality rates during the first month of exposure and were therefore subject to less crowded conditions. Although the authors stated that the differences in time to complete metamorphosis between treatment groups appeared to be a result of tank effects on relative growth rates rather than atrazine treatment; it may be more precise to conclude that tank effects obscured the study's ability to detect treatment effects. Given the confounding tank effects, it isn't possible for the authors to conclude that exposure to 10 and 25 µg/L atrazine does not consistently affect age, length, or weight of *R. clamitans* at metamorphic completion.

Because only about 24% of the tested organisms completed metamorphosis, and all of the analyses were conducted on juvenile organisms, the sampling strategy may have been biased and did not represent the population in the test.

While dihydroxytestosterone-treated frogs were identified as predominately (97.7%) male, the estradiol-treated frog sex ratio did not differ significantly from 50%. It is unclear whether the estradiol treatments, as a positive control, should have skewed sex ratios in favor of females; however, it is clear that the "treatment" did not impact sex ratios. Because hormone levels in the positive control were not measured, it is uncertain whether the lack of responsiveness is due to insufficient stimulus, poor water quality issues, or insensitivity of green frogs to estradiol treatments (i.e., green frogs represent a poor species for testing estrogenic responses). The authors stated that green frogs are not feminized when exposed to exogenous estradiol, but rather they are masculinized when exposed to exogenous androgens (*e.g.* DHT) and cite Foote and Witschi 1939. The fact that estradiol did not affect gonadal differentiation is inconsistent with previous studies, and it is not known if the frogs in this study were exposed to an efficacious dose of the hormone. .. In another study conducted by the same laboratory, estradiol concentrations in a static renewal system were less than 10% of the nominal target concentration. As a consequence, they did not observe the expected feminizing effects on *X. laevis*. This study with green frogs did not analyze estradiol concentrations, but they were certainly substantially below the target concentration given the static-renewal exposure regimen used.

According to Hayes (1998), estradiol treatment of *R. clamitans* did not affect sex ratio or produce mixed results (no effect on sex ratio to effects favoring either males or females); treatment of Ranids with testosterone produced primarily males.

Green frogs are a less studied experimental model than *X. laevis*. In *X. laevis*, the period of sensitivity toward femininization is during early prometamorphosis. This study was conducted in a manner that included the presumptive sensitive period of this species (*i.e.*, prometamorphosis).

Apparently there was some difficulty in identifying the presence of gonads in some of the animals, suggesting that the accuracy in detecting gonadal anomalies based on visual examinations (gross morphology) was somewhat limited.

The overall hypothesis tested was that waterborne concentrations of atrazine would not have an adverse effect on the gonads of the green frog (*Rana clamitans*) when exposed during the critical phases of development.

Based on an analysis of the raw atrazine measured concentration data (see attached SAS© [Statistical Analysis System, Release 8.01, Cary, North Carolina] and although only a limited number of analyses are reported on tank solutions, the tank atrazine concentrations ranged from 116 to 347% of mean-measured concentrations in stock solutions. On average, mean-measured concentrations (stock and tank solutions combined) contained 0.10 ± 0.016 µg/L; 11.76 ± 4.87 µg/L and 28.23 ± 8.47 µg/L in 0, 10 and 25 µg/L nominal exposure groups. Based on analyses conducted by Syngenta, exposure solutions averaged 0.14 ± 0.20 µg/L, 15.9 ± 6.7 µg/L, and 27.9 ± 8.88 µg/L. Although both sets of analyses tended to agree with one another, theyindicated that atrazine was present in the dilution water control and at levels that other studies have shown to result in gonadal developmental effects (Hayes *et al.* 2002*a* and 2002*b*).

Previous studies conducted by Hayes *et al.* 2002*a*,b showed effects as low as 0.1 µg/L; however, this study only used 10 and 25µg/L exposure levels. Also, Hayes' studies suggest that the incidence of gonadal effects was higher at lower doses. The effect on delayed time to metamorphosis and smaller size of metamorphs treated with 10 µg/L relative to both controls and animals treated with 25 µg/L may be reflective of a similar pattern.

G. <u>CONCLUSIONS</u>: Green frog (*Rana clamitans*) tadpoles reared from field-collected eggs were exposed for 273 days, beginning 5-days post-hatch, to two concentrations (10 and 25 µg/L) of atrazine. Positive controls, dihydroxytestosterone and 17-β estradiol (0.1 µg/mL in 0.005% ethanol), a negative control (water) and a solvent control (0.005% ethanol) were also run. Replicates (9) consisted of 30 free-swimming tadpoles each. Initially (exposure days 0 - 67) animals were maintained under static renewal conditions in 4 L of test solution; 50% tank changes were conducted every 72 hours. From Day 68 to Day 273, tadpoles were maintained in tanks containing 16L of test solution under static renewal. After 273 days exposures were terminated and tadpoles were maintained in a continuous flow-through 10-L glass tanks housed in large acrylic tanks containing 80 L of continuously renewing freshwater. At metamorphosis (fore-limb emergence) tadpoles were either housed individually or in small groups in 10-L glass tanks containing approximately 500 mL of freshwater.

Over the study period, mortality across all treatment groups averaged 76.5% and was attributed to poor water quality and overcrowding during the 273 day static-renewal phase of the study. While mean-measured concentrations of atrazine were relatively consistent with nominal values, measurements were made on freshly prepared stock solutions; hence it is unclear what atrazine concentrations were present in aged exposure solutions. Additionally, measurable levels of atrazine were detected in the negative controls. Although the concentrations of positive control hormones were not measured, the positive controls using dihydrotestosterone and 17-β estradiol suggested that green frogs only reacted to androgenic chemicals resulting in predominately (97.6%) male frogs, while the frogs were not affected by estradiol. It is uncertain whether this means that green frogs are unresponsive to estrogenic chemicals or whether there was sufficient estradiol in solution to elicit an effect. While no intersex (testicular and ovarian tissue in the same animal) was observed in any of the treatment groups, this observation was based on gross morphology and there were apparently difficulties in discerning the presence of gonads at all using this process. While time to and age at metamorphosis and the size of metamorphs were reduced in frogs treated with 10 μg/L atrazine, there was no difference in these same parameter for frogs treated with 25 μg/L atrazine relative to negative controls. Although there were no dose-dependent effects in green frogs related to atrazine treatment, only two concentrations were monitored.

Contrary to the GLP statement, this study represents an interim report and not a final report.

The high mortality indicative of poor water quality and overcrowding and the lack of response to the positive estradiol control made it difficult for the study authors to test the hypothesis that atrazine exposure was associated with developmental effects in amphibians. The study did provide the authors with a better appreciation for the conditions under which green frogs should be housed, and it suggests that the green frog may not be adequate for examining the effects of atrazine on amphibian development. The high mortality indicative of poor water quality and overcrowding and the lack of response to the positive estradiol controlmake it difficult to believe that this study was a sensitive indicator of the potential effects of atrazine on green frogs.

H. REFERENCES:

Foote, C. L. and E. Witschi 1939. Effect of sex hormones on the gonads of frog larvae (*Rana clamitans*): sex inversion in females; stability in males. The Anatomical Record 75(1): 75 - 83.

Hayes, T. B. 1998. Sex determination and primary sex differentiation in amphibians: genetic and developmental mechanisms. Journal of Experimental Zoology 281: 373 - 399.

Hayes, T. B., A. Collins, M. Lee, M. Mendoza, N. Noriega, A. S. Stuart, and A. Vonk. 2002a. Hermaphroditic, demasculinized frogs after exposure to the herbicide atrazine at low ecologically relevant doses. Proceedings of the National Academy of Sciences of the United States of America 99(8): 5476 - 5480.

Hayes, T. B., K. Haston, M. Tsui, A. Hoang, C. Haeffele, and A. Vonk. 2002b. Atrazine-induced hermaphroditism at 0.1 ppb in American leopard frogs (*Rana pipiens*): laboratory and field evidence. Environmental Health Perspectives.

AVERAGE MEAN MEASURED CONCENTRATION ACROSS TREATMENTS 1265

Obs	CONC	_TYPE_	_FREQ_	MEAN	STD	CV
1	0	0	30	0.1016	0.16001	157.557
2	10	0	28	11.7594	4.87077	41.420
3	25	0	27	28.2307	8.47165	30.009

PERCENT (PERC) OF ATRAZINE IN TANK RELATIVE TO STOCK SOLUTIONS 1266

Obs	CONC	_TYPE_	_FREQ_	STOCK	TANK	PERC
1	0	0	2	0.0872	0.3029	347.488
2	10	0	2	11.2688	18.1366	160.945
3	25	0	3	27.7255	32.2723	116.399

AVERAGE SYNGENTA MEAN MEASURED CONCENTRATIONS OF ATRAZINE 1267

Obs	CONC	_TYPE_	_FREQ_	MEAN2	STD	CV
1	0	0	18	0.1418	0.19528	137.680
2	10	0	10	15.9100	6.68937	42.045
3	25	0	15	27.9091	8.85104	31.714

COMPARISON OF MSU VERSUS SYNGENTA-MEASURED ATRAZINE CONCENTRATIONS AND PERCENTAGE RELATIVE 1268

Obs	CONC	MEAN	MEAN2	PERC
1	0	0.1016	0.1418	71.604
2	10	11.7594	15.9100	73.912
3	25	28.2307	27.9091	101.152

AVARAGE PERCENTAGE OF MALES AND FEMALES ACROSS ALL TREATMENTS (ACTUAL TREATMENTS NOT LISTED 1269

Obs	_TYPE_	_FREQ_	MALES	FEMALES	STD_M	STD_F
1	0	54	0.50648	0.49121	0.31086	0.30996

AVERAGE PERCENTAGE OF MALES BY TREATMENT GROUP 1052

Obs	GROUP	_TYPE_	_FREQ_	MEAN	STD	CV
1	10	0	9	38.9889	14.1583	36.3138
2	25	0	9	36.9388	20.4962	55.4870
3	CONTR	0	9	44.7173	32.7762	73.2965
4	DHT	0	9	98.2194	3.5370	3.6011
5	E2	0	9	35.6803	25.4366	71.2905
6	ETOH	0	9	41.2676	24.8757	60.2791

AVERAGE PERCENTAGE OF FEMALES BY TREATMENT GROUP 1053

Obs	GROUP	_TYPE_	_FREQ_	MEAN	STD	CV
1	10	0	9	61.0111	14.1583	23.206
2	25	0	9	63.0612	20.4962	32.502
3	CONTR	0	9	55.2827	32.7762	59.288
4	DHT	0	9	1.7806	3.5370	198.635
5	E2	0	9	64.3197	25.4366	39.547
6	ETOH	0	9	57.3435	24.6788	43.037

AVERAGE PERCENTAGE OF FEMALES BY TREATMENT GROUP 1054

Obs	GROUP	_TYPE_	_FREQ_	MEAN	STD	CV
1	10	0	9	0.00000	0.00000	.
2	25	0	9	0.00000	0.00000	.
3	CONTR	0	9	0.00000	0.00000	.
4	DHT	0	9	0.00000	0.00000	.
5	E2	0	9	0.00000	0.00000	.
6	ETOH	0	9	1.38889	3.92837	282.843

AVERAGE LENGTH OF FROGS BY GROUP 1055

Obs	GROUP	_TYPE_	_FREQ_	MEAN	STD	CV
1	10	0	77	2.38551	0.20537	8.6091
2	25	0	48	2.53903	0.26360	10.3818
3	Control	0	58	2.51361	0.29859	11.8789
4	DHT	0	104	2.41714	0.24883	10.2945
5	E2	0	40	2.56458	0.32778	12.7810
6	ETOH	0	69	2.46785	0.27089	10.9767

AVERAGE WEIGHT OF FROGS BY GROUP 1056

Obs	GROUP	_TYPE_	_FREQ_	MEAN	STD	CV
1	10	0	77	1.48109	0.34933	23.5857
2	25	0	48	1.64568	0.40205	24.4305
3	Control	0	58	1.75386	0.54678	31.1760
4	DHT	0	104	1.50077	0.45081	30.0385
5	E2	0	40	1.63576	0.51305	31.3650
6	ETOH	0	69	1.56208	0.43097	27.5892

AVERAGE AGE OF FROGS IN DAYS AT END OF STUDY BY GROUP 1057

Obs	GROUP	_TYPE_	_FREQ_	MEAN	STD	CV
1	10	0	77	376.703	58.6103	15.5587
2	25	0	48	342.135	47.8134	13.9750
3	Control	0	58	336.750	66.0096	19.6019
4	DHT	0	104	368.533	68.7472	18.6543
5	E2	0	40	342.152	26.5496	7.7596
6	ETOH	0	69	359.681	65.3223	18.1612

ANALYSIS OF VARIANCE FOR LENGTH OF FROGS BETWEEN GROUPS 1058

-- SEX=F --

The GLM Procedure

Class Level Information

Class	Levels	Values
GROUP	6	10 25 Control DHT E2 ETOH

Number of observations 153

NOTE: Due to missing values, only 135 observations can be used in this analysis.

Dependent Variable: LENGTH

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	5	0.90320512	0.18064102	2.47	0.0359
Error	129	9.44285747	0.07320045		
Corrected Total	134	10.34606259			

R-Square	Coeff Var	Root MSE	LENGTH Mean
0.087299	10.85520	0.270556	2.492407

Source	DF	Type I SS	Mean Square	F Value	Pr > F
GROUP	5	0.90320512	0.18064102	2.47	0.0359

Source	DF	Type III SS	Mean Square	F Value	Pr > F
GROUP	5	0.90320512	0.18064102	2.47	0.0359

ANALYSIS OF VARIANCE FOR LENGTH OF FROGS BETWEEN GROUPS 1060

--- SEX=M ---

The GLM Procedure

Class Level Information

Class	Levels	Values
GROUP	6	10 25 Control DHT E2 ETOH

Number of observations 194

NOTE: Due to missing values, only 162 observations can be used in this analysis.

Dependent Variable: LENGTH

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	5	0.43825553	0.08765111	1.37	0.2383
Error	156	9.97802868	0.06396172		
Corrected Total	161	10.41628421			

R-Square	Coeff Var	Root MSE	LENGTH Mean
0.042074	10.40884	0.252907	2.429728

Source	DF	Type I SS	Mean Square	F Value	Pr > F
GROUP	5	0.43825553	0.08765111	1.37	0.2383

Source	DF	Type III SS	Mean Square	F Value	Pr > F
GROUP	5	0.43825553	0.08765111	1.37	0.2383

PROC UNIVARIATE OUTPUT FOR RESIDUALS FROM GLM PROCEDURE USING LENGTH 1062

-- SEX=F --

The UNIVARIATE Procedure
Variable: Resid

Moments

N	135	Sum Weights	135
Mean	0	Sum Observations	0
Std Deviation	0.26546014	Variance	0.07046909
Skewness	0.22001009	Kurtosis	-0.3647052
Uncorrected SS	9.44285747	Corrected SS	9.44285747
Coeff Variation	.	Std Error Mean	0.02284717

Basic Statistical Measures

Location		Variability	
Mean	0.00000	Std Deviation	0.26546
Median	-0.02028	Variance	0.07047
Mode	-0.06828	Range	1.36128
		Interquartile Range	0.37773

NOTE: The mode displayed is the smallest of 5 modes with a count of 3.

Tests for Location: Mu0=0

Test	-Statistic-		-----p Value------	
Student's t	t	0	Pr > |t|	1.0000
Sign	M	-2.5	Pr >= |M|	0.7308
Signed Rank	S	-117.5	Pr >= |S|	0.7974

Tests for Normality

Test	--Statistic---		-----p Value------	
Shapiro-Wilk	W	0.989195	Pr < W	0.3771
Kolmogorov-Smirnov	D	0.053022	Pr > D	>0.1500
Cramer-von Mises	W-Sq	0.068716	Pr > W-Sq	>0.2500
Anderson-Darling	A-Sq	0.452489	Pr > A-Sq	>0.2500

Quantiles (Definition 5)

Quantile	Estimate
100% Max	0.720692
99%	0.631720
95%	0.420692
90%	0.363100
75% Q3	0.198425
50% Median	-0.020280
25% Q1	-0.179308
10%	-0.354575
5%	-0.382900
1%	-0.533280
0% Min	-0.640591

```
                     Extreme Observations

      ------Lowest------          ------Highest-----

          Value      Obs              Value      Obs

      -0.640591       58           0.453425       28
      -0.533280       84           0.531720       79
      -0.467280       85           0.620692      136
      -0.454900      120           0.631720       87
      -0.388280       98           0.720692      135


                        Missing Values

                                 -----Percent Of-----
      Missing                                 Missing
        Value        Count        All Obs        Obs

          .             18          11.76     100.00


PROC UNIVARIATE OUTPUT FOR RESIDUALS FROM GLM PROCEDURE USING LENGTH          1064

-------------------------------------------- SEX=F --------------------------------------------

                     The UNIVARIATE Procedure
                         Variable:  Resid

    Stem Leaf                          #             Boxplot
       7 2                             1                |
       6 23                            2                |
       5 3                             1                |
       4 222235                        6                |
       3 12222336777                  11                |
       2 0022223457779                13             +-----+
       1 02223355778899               14             |     |
       0 12222222245667777            17             |  +  |
      -0 988777766443332222           18             *-----*
      -1 9988887777766654433110       22             +-----+
      -2 88876555320                  11                |
      -3 988887775544443              15                |
      -4 75                            2                |
      -5 3                             1                |
      -6 4                             1                |
         ----+----+----+----+--
     Multiply Stem.Leaf by 10**-1


                      Normal Probability Plot
    0.75+                                                          *
        |                                                      * *++
        |                                                    *+++
        |                                                ****
        |                                           *****+
        |                                        ****+
        |                                    ****
    0.05+                               +****
        |                            ****
        |                       ******
        |                    ****
        |             ********
        |          *  *+++
        |    *++++
   -0.65+*++
         +----+----+----+----+----+----+----+----+----+----+
             -2        -1         0        +1        +2
```

PROC UNIVARIATE OUTPUT FOR RESIDUALS FROM GLM PROCEDURE USING LENGTH 1065

-- SEX=M --

The UNIVARIATE Procedure
Variable: Resid

Moments

N	162	Sum Weights	162
Mean	0	Sum Observations	0
Std Deviation	0.24894846	Variance	0.06197533
Skewness	0.31544092	Kurtosis	0.99990473
Uncorrected SS	9.97802868	Corrected SS	9.97802868
Coeff Variation	.	Std Error Mean	0.01955924

Basic Statistical Measures

Location		Variability	
Mean	0.000000	Std Deviation	0.24895
Median	0.003363	Variance	0.06198
Mode	0.103363	Range	1.46290
		Interquartile Range	0.32346

Tests for Location: Mu0=0

Test	-Statistic-		-----p Value------	
Student's t	t	0	Pr > |t|	1.0000
Sign	M	2	Pr >= |M|	0.8138
Signed Rank	S	-12.5	Pr >= |S|	0.9834

Tests for Normality

Test	--Statistic---		-----p Value------	
Shapiro-Wilk	W	0.979442	Pr < W	0.0164
Kolmogorov-Smirnov	D	0.037429	Pr > D	>0.1500
Cramer-von Mises	W-Sq	0.038106	Pr > W-Sq	>0.2500
Anderson-Darling	A-Sq	0.389134	Pr > A-Sq	>0.2500

Quantiles (Definition 5)

Quantile	Estimate
100% Max	0.9033625
99%	0.8605833
95%	0.3669048
90%	0.3000417
75% Q3	0.1603625
50% Median	0.0033625
25% Q1	-0.1630952
10%	-0.3090952
5%	-0.3996375
1%	-0.5374167
0% Min	-0.5595333

```
                         Extreme Observations

              ------Lowest------          ------Highest-----

                  Value      Obs              Value      Obs

              -0.559533       39           0.396042       22
              -0.537417      155           0.403363      134
              -0.533095      179           0.648363      121
              -0.513222       63           0.860583      166
              -0.489417      167           0.903363      101


                            Missing Values

                                     -----Percent Of-----
              Missing                             Missing
                Value       Count      All Obs       Obs

                  .            32        16.49     100.00
```

```
       PROC UNIVARIATE OUTPUT FOR RESIDUALS FROM GLM PROCEDURE USING LENGTH          1067

-------------------------------------------- SEX=M --------------------------------------------

                         The UNIVARIATE Procedure
                             Variable:  Resid

         Stem Leaf                         #             Boxplot
            9 0                            1                0
            8 6                            1                0
            7
            6 5                            1                0
            5
            4 00                           2                |
            3 000002557778                12                |
            2 012235555667788             15                |
            1 0000000222334455666678889   25             +-----+
            0 00012222335555666778888999  26             *--+--*
           -0 999998776654444332222111    24             |     |
           -1 87655433221111000           17             +-----+
           -2 9887755554444332110         19                |
           -3 776543100                    9                |
           -4 972000                       6                |
           -5 6431                         4                |
              ----+----+----+----+----+-
          Multiply Stem.Leaf by 10**-1


                          Normal Probability Plot
          0.95+                                                   *
              |                                                 *
              |
          0.65+                                               *   +
              |                                               ++++
              |                                           +++*
          0.35+                                       *******
              |                                   ****
              |                              *****
          0.05+                          *****
              |                     ******
              |                  +***
         -0.25+              *****
              |          ****
              |      +***
         -0.55+*+*+**
               +----+----+----+----+----+----+----+----+----+----+
                    -2        -1         0        +1        +2
```

```
PROC UNIVARIATE OUTPUT FOR RESIDUALS FROM GLM PROCEDURE USING LENGTH          1068

                         The UNIVARIATE Procedure
                             Variable:  Resid

                             Schematic Plots

      |
    1 +
      |
      |                        0
      |                        0
  0.8 +
      |
      |            |
      |            |           0
  0.6 +            |
      |            |
      |            |
      |            |
  0.4 +            |           |
      |            |           |
      |            |           |
      |            |           |
  0.2 +         +-----+        |
      |         |     |     +-----+
      |         |     |     |     |
      |         |     |     |     |
    0 +         *--+--*     *--+--*
      |         |     |     |     |
      |         |     |     |     |
      |         |     |     +-----+
 -0.2 +         +-----+        |
      |            |           |
      |            |           |
      |            |           |
 -0.4 +            |           |
      |            |           |
      |            |           |
      |            |           |
 -0.6 +            |
      |            |
      |
      |
 -0.8 +
        -----------+-----------+------------
  SEX                 F           M
```

NONPARAMETRIC COMPARISON OF FROG LENGTH ACROSS GROUPS 1069

--- SEX=F ---

The NPAR1WAY Procedure

Wilcoxon Scores (Rank Sums) for Variable LENGTH
Classified by Variable GROUP

GROUP	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
10	40	2141.00	2720.0	207.438789	53.525000
25	22	1699.50	1496.0	167.783300	77.250000
Control	25	1923.50	1700.0	176.467412	76.940000
DHT	2	178.50	136.0	54.883145	89.250000
E2	20	1549.00	1360.0	161.384599	77.450000
ETOH	26	1688.50	1768.0	179.142278	64.942308

Average scores were used for ties.

Kruskal-Wallis Test

Chi-Square	9.9388
DF	5
Pr > Chi-Square	0.0770

Median Scores (Number of Points Above Median) for Variable LENGTH
Classified by Variable GROUP

GROUP	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
10	40	14.0	19.851852	2.662548	0.350000
25	22	13.0	10.918519	2.153556	0.590909
Control	25	15.0	12.407407	2.265020	0.600000
DHT	2	1.0	0.992593	0.704444	0.500000
E2	20	11.0	9.925926	2.071427	0.550000
ETOH	26	13.0	12.903704	2.299353	0.500000

Average scores were used for ties.

Median One-Way Analysis

Chi-Square	5.4793
DF	5
Pr > Chi-Square	0.3602

NONPARAMETRIC COMPARISON OF FROG LENGTH ACROSS GROUPS 1071

-- SEX=M --

The NPAR1WAY Procedure

Wilcoxon Scores (Rank Sums) for Variable LENGTH
Classified by Variable GROUP

GROUP	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
10	24	1881.00	1956.00	211.993261	78.375000
25	15	1582.00	1222.50	172.974144	105.466667
Control	18	1456.00	1467.00	187.540207	80.888889
DHT	72	5359.50	5868.00	296.527103	74.437500
E2	12	1157.00	978.00	156.283506	96.416667
ETOH	21	1767.50	1711.50	200.445401	84.166667

Average scores were used for ties.

Kruskal-Wallis Test

Chi-Square	6.9456
DF	5
Pr > Chi-Square	0.2247

Median Scores (Number of Points Above Median) for Variable LENGTH
Classified by Variable GROUP

GROUP	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
10	24	12.0	12.00	2.267787	0.500000
25	15	11.0	7.50	1.850382	0.733333
Control	18	8.0	9.00	2.006202	0.444444
DHT	72	31.0	36.00	3.172083	0.430556
E2	12	9.0	6.00	1.671835	0.750000
ETOH	21	10.0	10.50	2.144254	0.476190

Average scores were used for ties.

Median One-Way Analysis

Chi-Square	7.8765
DF	5
Pr > Chi-Square	0.1632

ANALYSIS OF VARIANCE FOR WEIGHT OF FROGS BETWEEN GROUPS 1073

-- SEX=F --

The GLM Procedure

Class Level Information

Class	Levels	Values
GROUP	6	10 25 Control DHT E2 ETOH

Number of observations 153

NOTE: Due to missing values, only 134 observations can be used in this analysis.

Dependent Variable: WEIGHT

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	5	2.71751620	0.54350324	2.51	0.0330
Error	128	27.66481514	0.21613137		
Corrected Total	133	30.38233134			

R-Square	Coeff Var	Root MSE	WEIGHT Mean
0.089444	28.95896	0.464899	1.605373

Source	DF	Type I SS	Mean Square	F Value	Pr > F
GROUP	5	2.71751620	0.54350324	2.51	0.0330

Source	DF	Type III SS	Mean Square	F Value	Pr > F
GROUP	5	2.71751620	0.54350324	2.51	0.0330

ANALYSIS OF VARIANCE FOR WEIGHT OF FROGS BETWEEN GROUPS 1075

--- SEX=M ---

The GLM Procedure

Class Level Information

Class	Levels	Values
GROUP	6	10 25 Control DHT E2 ETOH

Number of observations 194

NOTE: Due to missing values, only 162 observations can be used in this analysis.

Dependent Variable: WEIGHT

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	5	0.79520647	0.15904129	0.86	0.5087
Error	156	28.80682131	0.18465911		
Corrected Total	161	29.60202778			

R-Square	Coeff Var	Root MSE	WEIGHT Mean
0.026863	27.92739	0.429720	1.538704

Source	DF	Type I SS	Mean Square	F Value	Pr > F
GROUP	5	0.79520647	0.15904129	0.86	0.5087

Source	DF	Type III SS	Mean Square	F Value	Pr > F
GROUP	5	0.79520647	0.15904129	0.86	0.5087

PROC UNIVARIATE OUTPUT FOR RESIDUALS FROM GLM PROCEDURE USING WEIGHT 1077

---------- SEX=F ----------

The UNIVARIATE Procedure
Variable: Resid

Moments

N	134	Sum Weights	134
Mean	0	Sum Observations	0
Std Deviation	0.45607689	Variance	0.20800613
Skewness	0.78265338	Kurtosis	0.6746575
Uncorrected SS	27.6648151	Corrected SS	27.6648151
Coeff Variation	.	Std Error Mean	0.03939904

Basic Statistical Measures

Location		Variability	
Mean	0.00000	Std Deviation	0.45608
Median	-0.07348	Variance	0.20801
Mode	0.30667	Range	2.42000
		Interquartile Range	0.60333

Tests for Location: Mu0=0

Test	-Statistic-		-----p Value------	
Student's t	t	0	Pr > |t|	1.0000
Sign	M	-12	Pr >= |M|	0.0465
Signed Rank	S	-382	Pr >= |S|	0.3983

Tests for Normality

Test	--Statistic---		-----p Value------	
Shapiro-Wilk	W	0.962472	Pr < W	0.0010
Kolmogorov-Smirnov	D	0.092468	Pr > D	<0.0100
Cramer-von Mises	W-Sq	0.224995	Pr > W-Sq	<0.0050
Anderson-Darling	A-Sq	1.348236	Pr > A-Sq	<0.0050

Quantiles (Definition 5)

Quantile	Estimate
100% Max	1.5448000
99%	1.2600000
95%	0.8948000
90%	0.5866667
75% Q3	0.2700000
50% Median	-0.0734848
25% Q1	-0.3333333
10%	-0.4933333
5%	-0.6261538
1%	-0.8336364
0% Min	-0.8752000

Extreme Observations

------Lowest------		------Highest-----	
Value	Obs	Value	Obs
-0.875200	84	0.993846	135
-0.833636	58	1.114800	79
-0.785200	98	1.206667	14
-0.765200	77	1.260000	124
-0.665200	88	1.544800	87

Missing Values

Missing Value	Count	-----Percent Of----- All Obs	Missing Obs
.	19	12.42	100.00

PROC UNIVARIATE OUTPUT FOR RESIDUALS FROM GLM PROCEDURE USING WEIGHT 1079

--- SEX=F ---

The UNIVARIATE Procedure
Variable: Resid

```
Stem Leaf                       #             Boxplot
  15 4                          1                0
  14
  13
  12 16                         2                0
  11 1                          1                |
  10                                             |
   9 69                         2                |
   8 79                         2                |
   7 13                         2                |
   6 68                         2                |
   5 13588899                   8                |
   4 014                        3                |
   3 111115777                  9                |
   2 124466678                  9             +-----+
   1 223369                     6             |     |
   0 12456789                   8             |  +  |
  -0 9997774322111000          16             *-----*
  -1 99765432211               11             |     |
  -2 977666554222100           15             |     |
  -3 887755433331              12             +-----+
  -4 9999655432110             13                |
  -5 733                        3                |
  -6 73322                      5                |
  -7 97                         2                |
  -8 83                         2                |
     ----+----+----+----+
 Multiply Stem.Leaf by 10**-1
```

```
              PROC UNIVARIATE OUTPUT FOR RESIDUALS FROM GLM PROCEDURE USING WEIGHT          1080

--------------------------------------------- SEX=F ---------------------------------------------

                                     The UNIVARIATE Procedure
                                         Variable:  Resid

                                          Normal Probability Plot
                     1.55+                                                  *
                         |
                         |
                         |                                              * *
                         |                                            *     +
                         |                                               +++
                         |                                          ** ++
                         |                                         **++
                         |                                        *++
                         |                                       *+
                         |                                    ***
                         |                                  +*
                     0.35+                                ***
                         |                              ***
                         |                            +**
                         |                         ++***
                         |                       +****
                         |                     +***
                         |                   ****
                         |                ****
                         |            *****
                         |          **+++
                         |       ****+
                         |    * * ++
                    -0.85+* *   ++
                          +----+----+----+----+----+----+----+----+----+----+
                              -2        -1         0        +1        +2
```

PROC UNIVARIATE OUTPUT FOR RESIDUALS FROM GLM PROCEDURE USING WEIGHT 1081

-- SEX=M --

The UNIVARIATE Procedure
Variable: Resid

Moments

N	162	Sum Weights	162
Mean	0	Sum Observations	0
Std Deviation	0.42299451	Variance	0.17892436
Skewness	1.66369399	Kurtosis	5.77981391
Uncorrected SS	28.8068213	Corrected SS	28.8068213
Coeff Variation	.	Std Error Mean	0.03323359

Basic Statistical Measures

Location		Variability	
Mean	0.00000	Std Deviation	0.42299
Median	-0.03063	Variance	0.17892
Mode	-0.22875	Range	2.79958
		Interquartile Range	0.44792

NOTE: The mode displayed is the smallest of 2 modes with a count of 4.

Tests for Location: Mu0=0

Test	-Statistic-		-----p Value------	
Student's t	t	0	Pr > |t|	1.0000
Sign	M	-6	Pr >= |M|	0.3875
Signed Rank	S	-649.5	Pr >= |S|	0.2788

Tests for Normality

Test	--Statistic---		-----p Value------	
Shapiro-Wilk	W	0.882296	Pr < W	<0.0001
Kolmogorov-Smirnov	D	0.095985	Pr > D	<0.0100
Cramer-von Mises	W-Sq	0.354957	Pr > W-Sq	<0.0050
Anderson-Darling	A-Sq	2.887157	Pr > A-Sq	<0.0050

Quantiles (Definition 5)

Quantile	Estimate
100% Max	2.051250
99%	1.881250
95%	0.491250
90%	0.377333
75% Q3	0.211250
50% Median	-0.030625
25% Q1	-0.236667
10%	-0.462500
5%	-0.572667
1%	-0.744762
0% Min	-0.748333

```
                    Extreme Observations

        ------Lowest------          -----Highest-----

            Value       Obs            Value       Obs

        -0.748333       167          1.20125       142
        -0.744762       179          1.28750        22
        -0.718750       108          1.40167       166
        -0.688333       155          1.88125       121
        -0.628750       147          2.05125       101

                       Missing Values

                                -----Percent Of-----
        Missing                              Missing
          Value       Count     All Obs          Obs

            .            32       16.49       100.00
```

```
PROC UNIVARIATE OUTPUT FOR RESIDUALS FROM GLM PROCEDURE USING WEIGHT          1083

----------------------------------------- SEX=M ------------------------------------------

                          The UNIVARIATE Procedure
                              Variable:  Resid

  Stem Leaf                                    #             Boxplot
    20 5                                       1                *
    18 8                                       1                *
    16
    14 0                                       1                0
    12 09                                      2                0
    10 8                                       1                0
     8
     6 1                                       1                |
     4 0223996                                 7                |
     2 0001112333455566779001455577888        31             +-----+
     0 012334455567899991122234456689         30             |  +  |
    -0 988776543322220009976665543322210      33             *-----*
    -2 977763220086666544333332221100         30             +-----+
    -4 87755110866633210                      17                |
    -6 5429300                                 7                |
       ----+----+----+----+----+----+---
   Multiply Stem.Leaf by 10**-1

                         Normal Probability Plot
       2.1+                                                        *
          |                                                      *
          |
          |                                                    *
          |                                                  **
          |                                                 *     ++
          |                                                  +++++
       0.7+                                              ++*+
          |                                          ++++****
          |                                      +********
          |                                  +******
          |                             *******
          |                         *******
          |                   ********+
      -0.7+*  *  ***++++
           +----+----+----+----+----+----+----+----+----+----+
               -2        -1         0        +1        +2
```

```
PROC UNIVARIATE OUTPUT FOR RESIDUALS FROM GLM PROCEDURE USING WEIGHT          1084

                    The UNIVARIATE Procedure
                        Variable:  Resid

                        Schematic Plots

     |
   2 +                        *
     |                        *
     |
     |
 1.5 +            0
     |                        0
     |            0           0
     |            |           0
   1 +            |
     |            |
     |            |           |
     |            |           |
 0.5 +            |           |
     |            |           |
     |         +-----+     +-----+
     |         |     |     |     |
   0 +         |  +  |     *--+--*
     |         *-----*     |     |
     |         |     |     +-----+
     |         +-----+        |
-0.5 +            |           |
     |            |           |
     |            |           |
     |            |
  -1 +
      ------------+-----------+-----------
 SEX                 F           M
```

NONPARAMETRIC COMPARISON OF FROG WEIGHT ACROSS GROUPS 1085

--- SEX=F ---

The NPAR1WAY Procedure

Wilcoxon Scores (Rank Sums) for Variable WEIGHT
Classified by Variable GROUP

GROUP	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
10	39	2190.50	2632.50	204.134406	56.166667
25	22	1584.00	1485.00	166.472482	72.000000
Control	25	2031.00	1687.50	175.067430	81.240000
DHT	2	195.00	135.00	54.490911	97.500000
E2	20	1388.00	1350.00	160.136203	69.400000
ETOH	26	1656.50	1755.00	177.713596	63.711538

Average scores were used for ties.

Kruskal-Wallis Test

Chi-Square	8.2408
DF	5
Pr > Chi-Square	0.1435

Median Scores (Number of Points Above Median) for Variable WEIGHT
Classified by Variable GROUP

GROUP	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
10	39	12.0	19.50	2.638993	0.307692
25	22	12.0	11.00	2.152110	0.545455
Control	25	17.0	12.50	2.263223	0.680000
DHT	2	2.0	1.00	0.704443	1.000000
E2	20	11.0	10.00	2.070197	0.550000
ETOH	26	13.0	13.00	2.297432	0.500000

Average scores were used for ties.

Median One-Way Analysis

Chi-Square	11.3060
DF	5
Pr > Chi-Square	0.0456

NONPARAMETRIC COMPARISON OF FROG WEIGHT ACROSS GROUPS 1087

--- SEX=M ---

The NPAR1WAY Procedure

Wilcoxon Scores (Rank Sums) for Variable WEIGHT
Classified by Variable GROUP

GROUP	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
10	24	1944.50	1956.00	212.080099	81.020833
25	15	1551.50	1222.50	173.044998	103.433333
Control	18	1709.00	1467.00	187.617028	94.944444
DHT	72	5186.00	5868.00	296.648568	72.027778
E2	12	1105.00	978.00	156.347523	92.083333
ETOH	21	1707.00	1711.50	200.527508	81.285714

Average scores were used for ties.

Kruskal-Wallis Test

Chi-Square	8.3092
DF	5
Pr > Chi-Square	0.1400

Median Scores (Number of Points Above Median) for Variable WEIGHT
Classified by Variable GROUP

GROUP	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
10	24	13.0	12.00	2.267787	0.541667
25	15	10.0	7.50	1.850382	0.666667
Control	18	13.0	9.00	2.006202	0.722222
DHT	72	29.0	36.00	3.172083	0.402778
E2	12	7.0	6.00	1.671835	0.583333
ETOH	21	9.0	10.50	2.144254	0.428571

Average scores were used for ties.

Median One-Way Analysis

Chi-Square	8.8182
DF	5
Pr > Chi-Square	0.1165

Data Evaluation Report on Response of *Xenopus laevis* to Atrazine Exposure: Assessment of the Mechanism of Action of Atrazine.

EPA MRID Number 458677-04

Data Requirement::

EPA DP Barcode D288775
Abcdef8

EPA MRID 458677-04
EPA Guideline 70-1(Special Study)

Test material: **Purity:** 97.1%

Common name Atrazine
chemical name: IUPAC
CAS name 6-chloro-N-ethyl-N'-(1-methylethyl)-1,3,5-triazine-2,4-diamine
CAS No. 1912-24-9
synonyms
EPA PC Code: 80803

Thomas M Steeger

Primary Reviewer: Thomas M. Steeger, Ph.D., Senior Biologist **Date:** March 27, 2003
Environmental Fate and Effects Division, ERB 4,
U. S. Environmental Protection Agency

Joseph E. Tietge

Secondary Reviewer(s): Joseph E. Tietge, M.S., Research Aquatic Biologist **Date:** April 16, 2003
Mid-Continent Ecology Division, National Health and Environmental Effects Research Laboratory (Duluth),
U. S. Environmental Protection Agency

Stephanie R. Irene

Stephanie Irene, Ph.D., Senior Advisor **Date:** 5/6/03
Environmental Fate and Effects Division, ERB 3, U. S. Environmental Protection Agency

Mary Frankenberry

Mary J. Frankenberry, Senior Statistician **Date:** 5/05/03
Environmental Fate and Effects Division, ERB 3, U. S. Environmental Protection Agency

EPA PC Code 080803

Date Evaluation Completed: 05/31/2003

CITATION: Hecker, M., K. K. Coady, D. L. Villeneuve, M. B. Murphy, P. D. Jones and J. P. Giesy. 2003. Response of *Xenopus laevis* to Atrazine Exposure: Assessment of the Mechanism of Action of Atrazine. Aquatic Toxicology Laboratory, Michigan State University, National Food Safety and Toxicology Center, E. Lansing, MI. Sponsor: Syngenta Crop Protection, Inc., Laboratory Study ID ECORISK Number MSU-04.

EXECUTIVE SUMMARY:

African clawed frogs (*Xenopus laevis*) were exposed (approximately 30 tadpoles/replicate with 8 replicates/treatment) to atrazine at 0.1, 1.0, 10, and 25 µg/L in FETAX media in a static renewal system where 50% of the exposure solutions were changed every 72 hours.. Exposures were also conducted using negative and positive (17-β estradiol and dihydrotestosterone) and a solvent (0.005% ethanol) control. Larvae were exposed from 72-hours post- hatch through metamorphosis (fore-limb emergence and tail resorption; NF stage 66). At metamorphosis, a subset (number not recorded) was euthanized; gonads were examined for gross morphology and gonad/larynx prepared for histology. The remaining animals were exposed until 2- 3 months post-metamorphosis. Afterwards, half the frogs were used for gross morphology and histology of the gonads. All frogs were examined for gross morphology, and 50 frogs per treatment were serially sectioned for gonad histology. One frog from each replicate tank (64 frogs total) was randomly selected, and blood (drawn by cardiac puncture), brain and gonads were collected for sex steroid hormone and aromatase activity assays. Plasma concentrations of testosterone and estradiol were measured by ELISA, while tritium-labeled water release assay was used to measure aromatase in brain and gonad tissue.

The study authors concluded that atrazine treatment did not affect mortality, time to metamorphosis, sex ratio, gonadal development, aromatase activity or steroid hormone plasma concentrations in a dose-dependent fashion. Also, estradiol (positive control) treatment only appeared to increase estradiol plasma concentrations. Dihyrotestosterone (positive control) increased larynx dilator muscle area in females, and neither positive control influenced sex ratios.

Although the most frequent gonadal abnormality based on gross morphology was discontinuous gonads, histology indicated that mixed sex/intersex (ovarian and testicular tissue in the same frog) was much more common than indicated by gross morphology. Since histology is still being conducted, it is premature to conclude that gonadal abnormalities were not treatment-related.

Poor water quality (elevated ammonia and nitrite with low dissolved oxygen) resulting from relatively high loading rates (30 tadpoles/4 liters of exposure solution) under static conditions may have compromised the growth and development of the test animals. On average, it took 73 days for frogs to complete metamorphosis and 17 (<2%) frogs in the study never underwent metamorphosis. Furthermore, the negative controls were contaminated with atrazine at levels comparable to those in the 0.1 µg/L atrazine treatment. High variability (coefficients of variability ranging as high as 524%) associated with gonadal aromatase activity and with plasma steroid hormone levels made it difficult to differentiate treatment effects. Also, it is unclear why estradiol treatment failed to skew sex ratios significantly in favor of females when other studies have demonstrated this effect. In summary, a combination of tank effects, contaminated controls, high variability and an apparent lack of responsiveness to estradiol made it difficult for the study authors to test their hypothesis and to differentiate treatment effects.

I. MATERIALS AND METHODS

GUIDELINE FOLLOWED: Nonguideline Study
COMPLIANCE: Not conducted under full GLP; however, most practices as defined by 40 CFR Part 160, August 19, 1989 were established for this study, including but not limited to:

- Written, authorized protocol
- Written, authorized Standard Operating Procedures for all key procedures.
- Organization and Personnel were sufficient in terms of number, education, training and experience.
- Facilities were of suitable size and construction
- Equipment used was of appropriate design and adequate capacity.
- Independent QA Inspections were conducted.
- Final Report was written
- Raw data, documentation, records, protocols, and final report were archieved.

A. MATERIALS:

1. Test Material Atrazine

Description: Not reported

Lot No./Batch No. : Not reported

Purity: 97.1%
Stability of compound undertest conditions: Not reported

Storage conditions of test chemicals:_ Not reported

2. Test organism:

Species: African clawed frog (*Xenopus laevis*)

Age at test initiation: Larvae (72-hours post-hatch)
Weight at study initiation: (mean and range) Not reported
Length at study initiation: (mean and range) Not reported

Source: Sexually mature X. laevis obtained from Xenopus Express® (Homossa, FL) induced with human chorionic gonadotropin; fertilized eggs dejellied in 2% L-cysteine in FETAX medium checked for viability, divided into groups of 35 fertilized eggs, then distributed into exposure solutions at 72-hours post-hatch.

B. STUDY DESIGN:

Objective:

1. To determine the effects of atrazine on metamorphosis and reproductive indices of larval *Xenopus laevis* were exposed from 72 hours after hatching until the completion of metamorphosis. Indices evaluated at the time of metamorphic completion included % initiation of metamorphosis, % completion of metamorphosis, time to metamorphosis, fresh body weight, snout-vent length, size of the laryngeal dilator muscle and gonad development.
2. To determine the concentration of circulating hormones, including testosterone and estradiol in control and atrazine-treated *X. laevis*
3. To investigate aromatase activity in the gonads and brain tissue of control and atrazine-exposed *X. laevis*.

1. Experimental Conditions

a) Range-finding Study: Exposure concentrations based on previous work

b. Definitive Study

Table 1 . Experimental Parameters

Parameter	Details
Acclimation: period: Conditions: (same as test or not) Feeding: Health: (any mortality observed)	 72 hours FETAX solution not reported not reported
Duration of the test	185-day study
Test condition static/flow- through Type of dilution system for flow-through method. Renewal rate for static renewal	 static renewal NA 50% test solution change every 72 hours
Aeration, if any	not reported

Parameter	Details
Test vessel Material: (glass/stainless steel) Size: Fill volume:	glass 10 L 4 L When frogs began to undergo metamorphosis, they were transferred to 40-L aquariums containing 10 L of test solution. According to the Protocol Changes/Revisions section of the report, frogs were maintained in 4-L of test solution until approximately one month post-metamorphosis, at which point they were transferred to larger aquariums.
Source of dilution water Quality:	Treated well water (MSU-University Research Containment Facility;
Water parameters: Hardness pH Dissolved oxygen Total oOrganic carbon Particulate Matter Ammonia Nitrite Metals Pesticides Chlorine Temperature {Salinity for marine or estuarine species} Intervals of water quality measurement	140 mg/L as $CaCO_3$ 7.7 (range 6.3 - 8.1) median DO 7.4 mg/L (low range: 2.5 - 8.8 mg/L) median total ammonia: 0.02 mg/L (range: 0.02 - 1.6 mg/L) median nitrite conc. 0.06 mg/L (range: 0.02 - 4.0 mg/L) 17 - 23°C (median temperature 20°C) NA not reported
Number of replicates/groups: negative control: FETAX media solvent control: 0.005% ethanol treated ones: atrazine at 0.1, 1.0, 10 and 25 µg/L in FETAX positive controls: dihydrotestosterone and estradiol in 0.005% EtOH.	 8 8 8 8

Parameter	Details
Number of organisms per replicate /groups: control: solvent control: treated ones:	 (30 tadpoles /rep) x 8 reps = 240 tadpoles 30 tadpoles /rep) x 8 reps = 240 tadpoles 30 tadpoles /rep) x 8 reps = 240 tadpoles
Biomass loading rate	30 tadpoles/4 L
Test concentrations: nominal: measured:	 0.1, 1.0, 10 and 25 µg/L 0.2, 1.0, 16, and 29 µg a.i./L
Solvent (type, percentage, if used)	FETAX for atrazine; 0.005% ethanol/FETAX for positive hormone controls FETAX: 0.625 g/L NaCl; 0.030 g/L KCl; 0.015 g/L $CaCl_2$; 0.096 g/L $NaHCO_3$; 0.06 g/L $CaSO_4*2H_2O$; and 0.075 g/L $MgSO_4$)
Lighting	not reported
Feeding	Appendix reports that frog brittle was previously analyzed (TTU-10/Syngenta Number 1833-01) by immunoassay yielding inconclusive results. Feeding regime is not reported
Recovery of chemical Level of Quantitation Level of Detection	ELISA (Envirogard Triazine®; Strategic Diagnostics Newark, DE)/Beacon Analytical triazine plate (Beacon Analytical Systems, Portland, ME) LOD 0.025 µg/L (Envirogard); 0.05 µg/L (Beacon)
Positive control {if used, indicate the chemical and concentrations}	dihydrotestosterone 0.1 µg/L 17-β estradiol 0.1 µg/L both hormones in 0.005% ethanol
Other parameters, if any	NA

2. Observations:

Table 2: Observations

Criteria	Details
Parameters measured including the sublethal effects/toxicity symptoms	mortality; time to metamorphosis, number completing metamorphosis, age (days) at metamorphosis, length, weight, gonadal abnormalities, sex
Observation intervals	daily
Were raw data included?	Yes

Other observations, if any	

Animals not reaching metamorphosis by 506 days were sacrificed.

All frogs completing metamorphosis were analyzed for gross morphology and histology of the gonads (no mention of kidneys).

At metamorphosis, a subset (number not recorded) was euthanized at metamorphic completion. Gonads were examined for gross morphology, and gonad/larynx was prepared for histology. Remaining tadpoles/metamorphs were transferred to 40-L aquariums containing 10 L of test solution where frogs were exposed until 2- 3 months post-metamorphosis. Afterwards, half the frogs were fixed in Bouin's solution and set aside for gross morphology and histology of the gonads. All frogs were examined for gross morphology, and 50 frogs per treatment were serially sectioned for gonad histology. The remaining half of the "grow-out" frogs were killed and necropsied from June 11 to June 24, 2002. One frog from each replicate tank (64 frogs total) was randomly selected and blood (drawn by cardiac puncture), brain and gonads were collected for sex steroid hormone and aromatase activity assays. Plasma concentrations of testosterone and estradiol were measured by ELISA. Tritium-labeled androstenedione water release assay was used to measure aromatase in brain and gonad tissue.

II. <u>RESULTS</u> and <u>DISCUSSION</u>:

Water Quality

No ammonia or nitrite levels are reported for February 6 through 21, 2002. Total ammonia nitrogen (range 0.02 - 1.6 mg/L) and nitrite (range: 0.02 - 3.0 mg/L) appeared to be highest during the January 10 through April 1, 2002. This period corresponds to roughly exposure days 21 through 80 and suggests that water quality may have influenced this study. Also, pH during the first week of the study (December 21 - 27, 2001) was unusually low (pH range: 6.3 - 6.9). The lower range of dissolved oxygen was consistently low from February 21 through March 20, 2002, averaging 4.9 mg/L and dropped as low as 2.5 mg/L.

Mean-measured concentrations of atrazine (**Table 3**) in the 0.1, 10, and 25 μg/L treatments ranged from 1.2 to 2.0 times higher than nominal concentrations, while the 1.0 μg/L group measured values were consistent with nominal. Atrazine was however detected at measurable levels in the dilution water control. Atrazine concentrations in the dilution water control ranged as high as those detected in the lowest (0.1μg/L) atrazine treatment. While atrazine was detected in positive controls and the solvent control, the levels reported are at and/or below the detection limit of the ELISA assay used for analysis.

Mortality

According to the study, total average observed mortality across all treatments was 16.1% (Table 4); however, there were many unaccounted animals and if they were dead, then the average mortality across treatments would be 20.2%.

A total of 17 surviving tadpoles did not initiate metamorphosis; therefore, 98.9% of surviving frogs initiated metamorphosis during the study period. There were no significant differences in age at completion of metamorphosis among treatment groups when treatments were compared to the appropriate controls (ANOVA atrazine data set, $p = 0.986$; ANOVA positive control data set, $p = 0.703$). The average age at metamorphic completion across all treatment groups was 72.8 days.

Growth

Average snout-vent length at completion of metamorphosis across all treatments was 1.85 cm; there was no difference in length between atrazine-treated and controls (ANOVA p= 0.066), nor was there a difference in weights compared to the positive controls (ANOVA p = 0.512). However, frogs in the solvent control were significantly (p = 0.032) longer (1.89 cm) than negative control frogs (1.75 cm).

Average weight of frogs at completion of metamorphosis across all treatments was 0.78 g. There was no significant difference in weight at completion of metamorphosis between treated and control animals (P = 0.22) or between treated and positive controls (p = 0.311). However, frogs in the solvent control (0.85 gm) were significantly different (p = 0.046) than negative controls (0.70 g).

Gonadal Abnormalities

Frogs were examined for gonadal deformities at two points along the course of the study; one subset was examined upon completion of metamorphosis (NF Stage 66), while the other set was examined 2 to 3 months after metamorphic completion (referred to as the "grow-out" frogs). Four types of gross gonadal abnormalities were observed: discontinuous gonad (abnormal segmentation of the gonad), intersex gonad (ovarian and testicular tissue separated left/right or rostral/caudal in a single individual), mixed sex gonad (co-occurrence of both ovarian and testicular tissue in a single gonad) and size irregularity (large size discrepancy between gonad pairs). Other abnormalities included small or underdeveloped ovaries (relatively few or no eggs). Although these effects were observed, no statistically significant treatment effects on the occurrence of gross gonadal abnormalities were found among NF Stage 66 frogs or among "grow-out" frogs.

The most common gross gonadal abnormality was discontinuous gonads for both NF Stage 66 (**Tables 5)** and grow-out frogs (**Table 6**). **Table 7** shows percentages of gross gonadal abnormalities for Stage 66 and grow-out frogs. However, the most common gonadal abnormality at a tissue level at Stage 66 was intersex. Since these evaluations are still underway, the actual percentage has yet to be determined. While there were no observations of intersex based on gross gonadal morphology of grow-out frogs, histology revealed a higher percentage of both mixed and intersex gonads, especially among males (**Tables 8** and **9**).

Sex Ratios

There were no consistent deviations from the expected 50:50 sex ratio (**Table 10**). While several tanks had statistically significant deviations from the 50:50 ratio, "the ratio was not consistently skewed in favor of one sex over the other, but varied from tank to tank." One ethanol and one dihydrotestosterone exposed tank and two 0.1 μg atrazine/L tanks had sex ratios in favor of more males, while one ethanol and one estradiol treated tank had skewed sex ratios in favor of more females. There was however no statistical difference between the percent males (ANOVA, p=0.108) or percent females (ANOVA, p=0.137) in atrazine-treated and negative controls. Also, there was no statistical difference between the percent of males (ANOVA, p=0.111) or females (ANOVA, p=0.232) in positive controls versus the solvent control. The estradiol group did have a greater percentage of "unknown" (sexually unidentifiable) frogs as compared to all other treatment groups except the 10 μg atrazine/L group.

Larynx Muscle

Overall, male frogs had laryngeal dilator muscle cross-sectional areas that were significantly greater than female muscle areas (Mann0Whitney U, p= 0.0001); there were no significant differences between male atrazine-treated frogs and negative controls (Kruskal-Wallis, p = 0.476). Male frogs exposed to DHT had greater laryngeal muscle area than "all other treatment groups" (**Table 11**). Female atrazine-treated frog laryngeal muscle area did not differ significantly from negative controls (Kruskal-Wallis, p = 0.181); however, females treated with DHT had greater laryngeal muscle area compared to "all other treatment groups" (Kruskal-Wallis, p = 0.0001) (**Table 12**).

Aromatase Activity

Aromatase activity in female gonads of juvenile *X. laevis* were significantly greater than in males (Mann0Whitney, p = 0.0001). There was no difference in activity of males (**Table 13**) treated with atrazine and controls (Kruskal-Wallis, p = 0.075), nor was there any difference between positive control males and solvent controls (Kruskal-Wallis, p = 0.382). There was no difference in female gonadal aromatase (**Table 14**) activity in atrazine-treated and control animals (Kruskal-Wallis, p = 0.821); however, females treated with estradiol had statistically less gonadal aromatase activity than solvent controls (Mann Whitney U, p = 0.0003).

Similarly brain aromatase activity was significantly greater (Mann-Whitney U, p=0.024) in females (mean = 8.9 x 10^2 fmol/h/mg protein) than in males (7.2 x 10^2 fmol/h/mg protein). There were no significant differences in male (**Table 15**) brain aromatase activity between atrazine-treated and control frogs (Kruskall-Wallis, p = 0.410). Estradiol-treated males had significantly higher activity than DHT-treated (Mann-Whitney, p = 0.012); however, neither positive control differed significantly from the solvent control. Additionally, there was no difference in activity between atrazine-treated females (**Table 16**) and controls (Kruskal Wallis, p = 0.885) nor among positive control females and solvent control (Kruskal-Wallis, p= 0.597).

Steroid Hormone Levels

While testosterone and estradiol were measurable in plasma, estradiol concentration in both male (**Table 17**) females (**Table 18**) were often less than the assay detection limit. Estradiol was significantly (Kruskal-Wallis, p= 0.02) higher in females (mean 4.2 ng/L) than in males (2.7 ng/L). Frogs treated with 1 µg/L atrazine had significantly less estradiol than controls, 0.1 and 25µg/L-treated frogs (Mann-Whitney, p < 0.015); however, males exposed to 0.1, 10 and 25 µg/L were not different than untreated controls. Plasma estradiol in males treated with estradiol were higher than those in the solvent control (Mann-Whitney U, p = 0.008)

Among female there was no difference in atrazine-treated and control frogs nor between positive control and solvent controls.

There were no differences between male and female testosterone levels (Kruskal-Wallis, p= 0.170); there was no significant difference between atrazine-treated males (**Table 19**) and untreated control males (Kruskal-Wallis, p = 0,270) nor between positive controls and solvent control (Kruskal-Wallis, p = 0.187). Likewise, there was no difference in plasma testosterone levels in atrazine-treated females (**Table 20**) and controls (Kruskal-Wallis, p = 0.179) or between positive controls and solvent controls (Kruskal-Wallis, p= 0.363).

C. REPORTED STATISTICS:

Table 3. Nominal versus mean-measured atrazine concentrations.

Treatment	Atrazine (nominal) µg/L	Syngenta mean-measured µg/L	MSU mean-measured µg/L
Control	--	0.16 (0.11 - 0.20)	0.11 (0.07 - 0.15)
Solvent Control	--	0.010 (0.01 - 0.01)	< 0.05
Dihydrotestosterone (0.1 µg/L)	--	0.020 (0.01 - 0.01)	< 0.05
17-β estradiol	--	0.04 (0.03 - 0.05)	< 0.05
0.1 µg/L	0.1	0.22 (0.17 - 0.26)	0.23 (0.16 - 0.31)
1.0 µg/L	1.0	1.0 (0.93)	1.4 (1.2 - 1.7)
10 µg/L	10	16 (14 - 19)	11 (9.7 - 13)
25 µg/L	25	29(24 - 35)	25 (22 - 28)

Table 4. Percent observed natural mortality, percent unaccounted for *X. laevis*, and total percent mortality (observed + unaccounted).

Treatment	Average % Natural Mortality (observed)	% Unaccounted Animals	Total % Mortality
Control	11.3	6.6	17.9
Solvent Control	14.4	3.4	17.8
Dihydrotestosterone (0.1 µg/L)	21.3	1.7	23.0
17-β estradiol	8.3	2.6	10.9
0.1 µg/L	20.4	3.4	23.8
1.0 µg/L	11.5	5.3	16.8
10 µg/L	18.7	3.1	21.8
25 µg/L	23.0	6.4	29.4

Table 5. Percent gross gonadal abnormalities in NF Stage 66 *X. laevis* exposed to negative control, solvent control, positive controls or various concentrations of atrazine.

Treatment	N	% Discontinuous Gonads	% Mixed Gonads	% Size Irregularities	% Intersex	% other
Control	45	2.2	0.0	2.2	0.0	2.2
Solvent Control	45	0.0	0.0	0.0	0.0	2.2
Dihydrotestosterone (0.1 µg/L)	42	4.8	2.4	2.4	0.0	4.78
17-β estradiol	46	6.5	4.4	0.0	2.2	2.2
0.1 µg/L	40	5.0	0.0	0.0	0.0	7.5
1.0 µg/L	46	2.2	0.0	2.2	0.0	0.0
10 µg/L	43	7.0	0.0	0.0	0.0	4.7
25 µg/L	39	5.1	2.6	0.0	0.0	0.0

Table 6. Percent gross gonadal abnormalities in male and female grow-out *X. laevis* exposed to negative control, solvent control, positive controls or various concentrations of atrazine.

Treatment	N	% Discontinuous Gonads	% Mixed Gonads	% Size Irregularities
Control	75	1.4	0.0	2.7
Solvent Control	75	2.7	0.0	1.3
Dihydrotestosterone (0.1 µg/L)	72	1.4	0.0	0.0
17-β estradiol	77	2.6	0.0	0.0
0.1 µg/L	71	4.2	0.0	0.0
1.0 µg/L	79	1.3	0.0	2.5
10 µg/L	73	4.1	2.7	0.0
25 µg/L	67	3.0	0.0	0.0

Table 7. Percent gross gonadal abnormalities in Stage 66 and male and female grow-out *X. laevis* exposed to negative control, solvent control, positive controls or various concentrations of atrazine.

Treatment	N	% Discontinuous Gonads	% Mixed Gonads	% Size Irregularities
Control	120	3.6	0.0	4.9
Solvent Control	120	2.7	0.0	1.3
Dihydrotestosterone (0.1 µg/L)	114	6.2	2.4	2.4
17-β estradiol	123	9.1	6.6	0.0
0.1 µg/L	111	9.2	0.0	0.0
1.0 µg/L	125	3.5	0.0	4.7
10 µg/L	116	11.1	2.7	0.0
25 µg/L	106	8.1	2.6	0.0

Table 8. Percent gonadal abnormalities at the tissue level (based on histology) in grow-out male *X. laevis* exposed to negative control, solvent control, positive controls or various concentrations of atrazine.

Treatment	N	% Mixed Sex	% Intersex	% Other Abnormalities
Control	25	8.0	16.0	0.0
Solvent Control	25	20.0	4.0	0.0
Dihydrotestosterone (0.1 µg/L)	25	4.0	0.0	0.0
17-β estradiol	25	32.0	0.0	0.0
0.1 µg/L	25	16.0	0.0	0.0
1.0 µg/L	25	8.0	4.0	0.0
10 µg/L	25	12.0	0.0	0.0
25 µg/L	25	8.0	0.0	0.0

Table 9. Percent gonadal abnormalities at the tissue level (based on histology) in grow-out female *X. laevis* exposed to negative control, solvent control, positive controls or various concentrations of atrazine.

Treatment	N	% Mixed Sex	% Intersex	% Other Abnormalities
Control	25	0.0	0.0	0.0
Solvent Control	25	0.0	0.0	4.0
Dihydrotestosterone (0.1 µg/L)	25	0.0	0.0	0.0
17-β estradiol	25	8.0	0.0	0.0
0.1 µg/L	24	0.0	0.0	0.0
1.0 µg/L	25	0.0	0.0	8.0
10 µg/L	25	0.0	0.0	0.0
25 µg/L	25	0.0	0.0	4.0

Table 10. Percent male and female grow-out *X.. laevis* in each treatment.

Treatment	% Males	% Females	% Unkown
Control	48.2	51.8	0.0
Solvent Control	45.2	54.8	0.0
Dihydrotestosterone (0.1 µg/L)	50.5	49.5	0.0
17-β estradiol	36.2	61.3	2.5
0.1 µg/L	57.9	42.1	0.0
1.0 µg/L	50.7	49.3	0.0
10 µg/L	49.2	49.7	1.1
25 µg/L	42.5	56.9	0.6

Table 11. Layrngeal dilator muscle areas for male *X. laevis* exposed to positive controls and various concentrations of atrazine.

Treatment	Mean Left Laryngeal Muscle Area (mm^2)	Standard Error	Mean Right Laryngeal Muscle Area (mm^2)	Standard Error
Control	2.1×10^{-1}	9.0×10^{-3}	2.1×10^{-1}	1.0×10^{-2}
Solvent Control	1.9×10^{-1}	2.0×10^{-2}	1.7×10^{-1}	1.6×10^{-2}
Dihydrotestosterone (0.1 µg/L)	3.0×10^{-1}*	2.2×10^{-2}	3.1×10^{-1}*	2.1×10^{-2}
17-β estradiol	2.2×10^{-1}	2.3×10^{-2}	2.1×10^{-1}	2.0×10^{-2}
0.1 µg/L	2.1×10^{-1}	2.4×10^{-2}	2.2×10^{-1}	2.8×10^{-2}
1.0 µg/L	2.3×10^{-1}	1.1×10^{-2}	2.2×10^{-1}	8.0×10^{-3}
10 µg/L	2.2×10^{-1}	1.3×10^{-2}	2.2×10^{-1}	1.8×10^{-2}
25 µg/L	2.0×10^{-1}	1.3×10^{-2}	1.9×10^{-1}	3.0×10^{-2}

*significantly different (Kruskal-Wallis, p =0.0001)

Table 12. Layrngeal dilator muscle areas for female *X. laevis* exposed to positive controls and various concentrations of atrazine.

Treatment	Mean Left Laryngeal Muscle Area (mm^2)	Standard Error	Mean Right Laryngeal Muscle Area (mm^2)	Standard Error
Control	1.5×10^{-1}	1.0×10^{-2}	1.5×10^{-1}	1.0×10^{-2}
Solvent Control	1.9×10^{-1}	2.8×10^{-2}	1.9×10^{-1}	3.6×10^{-2}
Dihydrotestosterone (0.1 µg/L)	3.5×10^{-1}*	6.8×10^{-2}	3.6×10^{-1}*	7.2×10^{-2}
17-β estradiol	1.5×10^{-1}**	8.0×10^{-3}	1.5×10^{-1}	1.3×10^{-2}
0.1 µg/L	2.1×10^{-1}	2.5×10^{-2}	2.1×10^{-1}	2.8×10^{-2}
1.0 µg/L	1.7×10^{-1}	1.2×10^{-2}	1.6×10^{-1}	1.4×10^{-2}
10 µg/L	1.5×10^{-1}	1.3×10^{-2}	1.7×10^{-1}	3.6×10^{-2}
25 µg/L	1.5×10^{-1}	7.0×10^{-3}	1.5×10^{-1}	8.0×10^{-2}

*significantly different (Kruskal-Wallis, p =0.0001)
** value corrected to 1.5×10^{-1} rather than 15×10^{-1} as reported in study; assumed to be a typo.

Table 13. Gonadal aromatase activity in male juvenile *X. laevis* exposed to positive controls and various concentrations of atrazine.

Treatment	N	Male Mean Gonad Aromatase Activity (fmol/h/mg protein)	Male Median Gonad Aromatase Activity (fmol/h/mg protein)	Standard Error	Coefficient of Variation
Control	19	8.9	0.0	4.2	206%
Solvent Control	21	5.6	0.0	2.7	221%
Dihydrotestosterone (0.1 µg/L)	21	7.2	0.0	3.2	204%
17-β estradiol	25	1.3×10^{-1}	0.0	4.2	161%
0.1 µg/L	24	8.7	0.0	4.7	264%
1.0 µg/L	23	3.6×10^{-1}	0.0	2.9	386%
10 µg/L	18	1.9	0.0	1.9	424%
25 µg/L	21	0.9	0.0	0.9	457%

Table 14. Gonadal aromatase activity in female juvenile *X. laevis* exposed to positive controls and various concentrations of atrazine.

Treatment	N	Female Mean Gonad Aromatase Activity (fmol/h/mg protein)	Female Median Gonad Aromatase Activity (fmol/h/mg protein)	Standard Error	Coefficient of Variation
Control	16	530	270	160	121%
Solvent Control	17	610	400	130	88%
Dihydrotestosterone (0.1 µg/L)	15	200	110	52	101%
17-β estradiol	15	110*	380	45	158%
0.1 µg/L	10	330	230	120	115%
1.0 µg/L	15	490	210	160	127%
10 µg/L	18	510	330	120	100%
25 µg/L	16	500	350	110	88%

*significantly different (Kruskal-Wallis, p =0.0003)

Table 15. Brain aromatase activity in male juvenile *X. laevis* exposed to positive controls and various concentrations of atrazine.

Treatment	N	Male Mean Brain Aromatase Activity (fmol/h/mg protein)	Male Median Brain Aromatase Activity (fmol/h/mg protein)	Standard Error	Coefficient of Variation
Control	18	0.71	0.54	0.12	72%
Solvent Control	21	0.93	0.89	0.17	84%
Dihydrotestosterone (0.1 µg/L)	19	0.54	0.47	0.09	73%
17-β estradiol	24	1.1	0.91	0.16	71%
0.1 µg/L	24	0.58	0.55	0.09	76%
1.0 µg/L	22	0.71	0.47	0.15	99%
10 µg/L	18	0.43	0.27	0.10	99%
25 µg/L	21	0.80	0.81	0.16	92%

Table 16. Brain aromatase activity in female juvenile *X. laevis* exposed to positive controls and various concentrations of atrazine.

Treatment	N	Female Mean Brain Aromatase Activity (fmol/h/mg protein)	Female Median Brain Aromatase Activity (fmol/h/mg protein)	Standard Error	Coefficient of Variation
Control	15	1.1	0.81	0.22	77%
Solvent Control	15	0.81	0.70	0.12	57%
Dihydrotestosterone (0.1 µg/L)	15	0.63	0.46	0.11	68%
17-β estradiol	15	0.86	0.48	0.20	90%
0.1 µg/L	10	0..93	0.85	0.22	75%
1.0 µg/L	16	0.95	0.86	0.17	72%
10 µg/L	18	0.98	0.90	0.16	69%
25 µg/L	16	0.94	0.59	0.23	98%

Table 17. Plasma estradiol concentrations in male juvenile *X. laevis* exposed to positive controls and various concentrations of atrazine.

Treatment	N	Male Mean Estradiol (ng/mL)	Male Median Estradiol (ng/mL)	Standard Error	Coefficient of Variation
Control	20	3.5	0.4	1.5	192%
Solvent Control	21	0.6	0.03	0.5	382%
Dihydrotestosterone (0.1 µg/L)	21	1.9	0.2	0.6	145%
17-β estradiol	25	6.9**	0.4	5.8	420%
0.1 µg/L	26	1.3	0.1	0.4	157%
1.0 µg/L	24	0.029*	0.024	3.1	524%
10 µg/L	20	5.8	0.1	4.0	308%
25 µg/L	23	1.8	0.4	0.5	133%

* significantly different (Mann-Whitney U, p = 0..015)

** significantly different (Mann-Whitney U, p = 0.008)

Table 18. Plasma estradiol concentrations in female juvenile *X. laevis* exposed to positive controls and various concentrations of atrazine.

Treatment	N	Female Mean Estradiol (ng/mL)	Female Median Estradiol (ng/mL)	Standard Error	Coefficient of Variation
Control	15	9.8	0.2	6.0	237%
Solvent Control	18	0.066	0.024	0.022	141%
Dihydrotestosterone (0.1 µg/L)	16	15	0.035	15	400%
17-β estradiol	15	2.2	0.2	0.9	158%
0.1 µg/L	12	4.2	0.017	2.6	214%
1.0 µg/L	16	0.5	0.027	0.3	240%
10 µg/L	17	0.9	0.018	0.9	412%
25 µg/L	17	1.2	0.032	0.6	206%

Table 19. Plasma testosterone concentrations in male juvenile *X. laevis* exposed to positive controls and various concentrations of atrazine.

Treatment	N	Male Mean Testosterone (ng/mL)	Male Median Testosterone (ng/mL)	Standard Error	Coefficient of Variation
Control	20	1.7	0.1	0.8	210%
Solvent Control	21	0.6	0.1	0.4	306%
Dihydrotestosterone (0.1 µg/L)	21	0.3	0.2	0.1	153%
17-β estradiol	25	1.2	0.2	0.8	333%
0.1 µg/L	26	0.4	0.1	0.1	127%
1.0 µg/L	24	0.1	0.1	0.018	88%
10 µg/L	20	1.6	0.2	1.1	307%
25 µg/L	23	0.3	0.1	0.1	160%

Table 20. Plasma testosterone concentrations in female juvenile *X. laevis* exposed to positive controls and various concentrations of atrazine.

Treatment	N	Female Mean Testosterone (ng/mL)	Female Median Testosterone (ng/mL)	Standard Error	Coefficient of Variation
Control	15	2.5	0.1	1.3	201%
Solvent Control	18	0.1	0.1	0.03	127%
Dihydrotestosterone (0.1 µg/L)	16	2.6	0.1	2.4	369%
17-β estradiol	15	0.7	0.1	0.4	221%
0.1 µg/L	12	2.4	0.1	1.7	245%
1.0 µg/L	16	0.1	0.1	0.023	92%
10 µg/L	17	0.3	0.1	0.2	275%
25 µg/L	17	0.2	0.1	0.1	206%

D. <u>VERIFICATION OF STATISTICAL RESULTS</u>: Basic analyses run using SAS® (Statistical Analysis System, Release 8.01, Cary, North Carolina); see attached printout.

E. STUDY DEFICIENCIES: The feeding regime is not reported; however, the animals were apparently fed frog brittle. The appendix reports that a previous immunoassay of the food was "inconclusive". It is unclear what "inconclusive" refers to; however, an analysis of the current study's food supply was apparently not run. Also, an immunoassay would not provide information on a broad range of contaminants and suggests that the food analysis may have only looked for atrazine residues.

Atrazine was detected in the negative control. Apparently tanks were not covered and animals may have hopped between treatments.

Water quality parameters in terms of total ammonia and nitrite were unusually high, while dissolved oxygen dropped very low.

F. REVIEWER'S COMMENTS:

From January 10 through April 1, 2002, water quality parameters, *i.e.*, total ammonia nitrogen, nitrite and dissolved oxygen, suggest that water quality may have been unreasonably poor. Total ammonia nitrogen and nitrite rose as high as 1.6 mg/L and 3 mg/L, respectively, while dissolved oxygen dropped as low as 2.5 mg/L. In some cases, *i.e.*, February 6, 2002, dissolved oxygen concentrations were low across all replicate tanks over 5 out of the 8 treatments. At a median temperature of 20°C, the solubility of oxygen is 8.84 mg/L (Boyd 1984); thus, at dissolved oxygen concentrations of 2.5 mg/L, the water is roughly 28% of saturation In a study of *Rana clamitans* initiated in August 2001 (ECORISK Number MSU-03) by the same group of researchers, a similar stocking rate of 30 tadpoles per 4 L of exposure solution resulted in sufficiently poor water quality to impair the survival/development of the tadpoles; the researchers concluded that "to limit mortality and maximize development in future laboratory exposures with *R. clamitans*, it is recommended that tadpoles be reared at low densities in static tanks (< 1 tadpole/L) or in a continuous flow-through system." Mortality in the current study averaged 20% across all treatments; however, no data are provided to determine whether mortality was associated with the period of poor water quality. The similar stocking rate for *X. laevis* is probably responsible for the poor water quality during a considerable portion (80 days) of the developmental/growth period for these tadpoles.

When frogs began to undergo metamorphosis, they were transferred to 40-L aquariums containing 10 L of test solution. According to the Protocol Changes/Revisions section of the report, frogs were maintained in 4-L of test solution until approximately one month post-metamorphosis, at which point they were transferred to larger aquariums. However, when the frogs were in various stages of metamorphosis, the 10-L tanks containing 4 L of test solution was partitioned into three sections. One section housed pre-metamorphic tadpoles, one section held metamorphosing tadpoles and froglets, and the third section contained post-metamorphic frogs. This was done to prevent predation on smaller-sized tadpoles and to facilitate enumeration of individuals in each stage of development. While the tanks may have been larger, the partitioning may have crowded the animals.

Atrazine was apparently present in negative control samples at levels comparable to the 0.1 µg/L atrazine treatment concentration level. Additionally, although mean-measured concentrations indicate that atrazine treatment levels were similar or even higher than nominal, the data represent an average of freshly prepared stock solutions and 72-hour aged exposure solutions. It would have been useful to know atrazine levels in the exposure solutions at 72 hours before the solutions were renewed.

The authors dismissed the apparent atrazine contamination of the controls. However, 21 of 49 measurements exceeded 0.1 ug/L, the nominal concentration of the lowest test concentration tested. And although the actual

concentrations in the 0.1 ug/L treatments were on average higher than nominal, 10 of the control concentrations exceeded the concentrations measured on the same day in the 0.1 ug/L treatment. This suggests that these exposures were probably not different. The author notes in the discussion that contaminants in surface waters can give false positives due to cross reactivity with the antibody used in the analysis. However, it should also be noted that they used well water that was treated with reverse osmosis.
While it is technically true that the controls are indistinguishable from the low test concentration, it is also evident that the animals did not show any dose dependent effects from atrazine over the concentrations tested.

In terms of exposure, in general the 50% renewal every 72 hours is not optimal, and probably explains the lack of effects in the "positive controls." Exposure to these compounds may not be efficacious based on mass limitations of the compound and sorption to organic matter and system surfaces. This exposure protocol is probably also responsible for the mortality rates of approximately 20% and may explain the relatively long time to metamorphosis.

The percentage of grow-out frogs with gonadal deformities based on gross morphology was considerably different than estimates based on histology. Since the histological analysis is still being conducted on both groups of frog gonad samples, the data presented in this report are preliminary and inconclusive. Based on preliminary data, it appears that the incidence of mixed/intersex animals was highest in the estradiol positive control (32%); however, both the negative and solvent controls had relatively high incidence (24%) of mixed/intersex animals. Because negative control animals were apparently exposed to levels of atrazine consistent with those in the 0.1 µg/L atrazine treatment, the relatively high incidence of mixed/intersex tissue in male gonads (negative control = 24%) cannot be considered reflective of background. The ability of this study to discriminate treatment effects appears compromised. Additionally, males were clearly more prone to mixed/intersex phenomena because only females treated with estradiol demonstrated the effect (8%) based on histological analysis.

The utility of the positive controls is uncertain. Previous studies (Chang and Witschi 1955*a*, *b*, Gallien 1953, Gallien 1954, Gallien 1957, Hayes *et al.* 2002*a*, *b*) suggested that treatment with estradiol would skew *X. laevis* sex ratios in favor of females at estradiol concentrations of 0.1 µg/L; however, at lower estradiol concentrations, *i.e.*, 0.04 µg/L estradiol may not impact *Xenopus* sex ratios (Chang and Witschi 1955*a*, *b*). In this study, the authors report that there "were no significant differences in the % females or % males among the positive controls (ANOVA, p=0.111 and 0.232, respectively" and therefore, 0.1 µg/L of estradiol had no apparent affect on sex ratios. It is unclear whether the unresponsiveness of *X. laevis* to this steroid is reflective of the frog's genuine lack of sensitivity (contrary to the Hayes' study) or if the hormone levels were not sufficiently high enough to result in a significant effect on sex ratios. Dihydrotestosterone only appeared to affect laryngeal dilator muscle areas . Although water samples were collected from positive controls, hormone concentrations were not reported.

Gonad aromatase levels in females were generally two orders of magnitude greater than in males. Although gonad aromatase activity in both atrazine-treated males and females was not significantly different from controls, it is clear from **Tables 13** and **14** that there was considerable variability associated with these estimates. The median value for males across all treatments was 0.0 fmol/h/mg protein, while mean values ranged from 0.9 to 36 fmol/h/mg protein. Coefficients of variability (CV = [standard deviation ÷ mean]*100) ranged from 264% to 457%. It is interesting to note that the CV for atrazine-treated males is positively correlated (r = 0.97) with the concentration of atrazine.

Brain aromatase levels, while statistically higher in females than in males, were within the same order of magnitude and were generally less variable (CV range: 69 - 99%) than gonadal aromatase levels. Once again, the highest variability in male aromatase activity was associated with atrazine-treated animals.

The measurement of aromatase activity is conducted at a lifestage that is not relevant to gonadal differentiation, so these measurements are not very useful. Furthermore, it is still an open question as to whether atrazine can, in fact, induce aromatase activity because there are no efforts to evaluate the time course of activity in the presence of atrazine exposure. Since aromatase activity is part of a homeostatically controlled system, it is possible that there could be transient perturbation in activity that subsides upon the system reaching homeostasis. Because there are no apparent changes in gonadal differentiation, though, this is probably a moot point.

The high variability of the aromatase activity in male gonads may be caused by the relatively high rate of "mixed sex" and "intersex" tissues as determined by histological measurements. Ovaries and brains from both sexes had substantially lower variances associated with the aromatase measurements.

This study does include exposure to the appropriate life stages to evaluate the effects of atrazine on gonadal differentiation, notably stages 44-54, which are apparently the most sensitive to femininization by exogenous estrogenic chemicals.

Plasma steroid hormone levels showed significantly less estradiol in males treated with 1 µg/L atrazine; however, none of the other atrazine treated animals were significantly different than controls. Estradiol treated frogs had significantly higher estradiol than the solvent controls. Similar to the gonad aromatase assay, the variability associated with the plasma estradiol and testosterone were considerable with CVs ranging from 133% to 524% and 88% to 369%, respectively. Given this level of variability and the fact that many of the frogs tested had plasma steroid levels at or near the detection limit of the assay, jevenile frog plasma steroid levels may not be a reliable measure of atrazine-treatment effects. Although an effort was made to draw blood samples within a 3-hour window during the night, the fact that this process continued over a number of days may have confounded the study.

In the entire experiment, 17 surviving tadpoles did not initiate metamorphosis by 506 days and were apparently terminated. It is unclear whether these animals were necropsied or what the status of their gonads was.

G. CONCLUSIONS:

Atrazine contamination of the negative controls and the lack of responsiveness to the positive estradiol control limited the value of this study in differentiatingtreatment effects. In addition, high variability in gonad aromatase activity and plasma activity and plasma estradiol and testosterone concentrations made it difficult to test the hypothesis of this study. Mortality was relatively consistent (mean = 20%) across all exposure groups with approximately 5% of the mortality presumably due to cannabalism. Poor water quality (high ammonia/nitrite and low dissolved oxygen) probably resulted from relatively high stocking rates (30 tadpoles in 4 L of static exposure solution) in which only 50% of the exposure solution was changed every 72 hours. More rigorous laboratory testing is needed to characterize the effects of atrazine on amphibian development.

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AVERAGE LENGTH AND WEIGHT OF MALE AND FEMALE FROGS BY TREATMENT 128

Obs	Treat	Sex	_TYPE_	_FREQ_	WEIGHT	LENGTH	W_SD	L_SD
1	0	F	0	73	3.21890	3.00730	0.79658	0.27341
2	0	M	0	73	3.04475	2.95132	0.82616	0.25585
3	0.1	F	0	56	3.40339	3.12521	1.10743	0.73651
4	0.1	M	0	82	3.40073	3.03395	0.91877	0.27963
5	1	F	0	77	2.83974	2.85431	0.91108	0.30657
6	1	M	0	80	2.86188	2.86024	0.75054	0.23584
7	10	F	0	70	3.26000	2.96167	1.10588	0.30883
8	10	M	0	68	3.10881	2.94512	0.75772	0.24669
9	10	U	0	2	3.43000	3.02150	1.10309	0.29345
10	25	F	0	74	3.61644	3.10945	1.15980	0.32159
11	25	M	0	55	3.16273	2.98069	0.91015	0.31387
12	DHT	F	0	69	3.22638	2.94362	1.72071	0.41002
13	DHT	M	0	72	3.25056	2.98472	1.31500	0.37757
14	E2	F	0	89	3.05114	3.03123	0.76144	0.26143
15	E2	M	0	62	2.76694	2.93268	0.65439	0.27893
16	E2	U	0	2	2.75500	2.97600	0.81317	0.28567
17	ETOH	F	0	80	3.26163	2.99979	0.95336	0.31710
18	ETOH	M	0	63	3.26365	3.01614	0.92449	0.31516

ANOVA FOR WEIGHT OF FROGS ACROSS TREATMENT BY SEX 129

--- Sex=F ---

The ANOVA Procedure

Class Level Information

Class	Levels	Values
Treat	5	0 0.1 1 10 25

Number of observations 40

Dependent Variable: WEIGHT

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	4	3.66900699	0.91725175	2.22	0.0863
Error	35	14.43121687	0.41232048		
Corrected Total	39	18.10022385			

R-Square	Coeff Var	Root MSE	WEIGHT Mean
0.202705	19.12619	0.642122	3.357291

Source	DF	Anova SS	Mean Square	F Value	Pr > F
Treat	4	3.66900699	0.91725175	2.22	0.0863

Levene's Test for Homogeneity of WEIGHT Variance
ANOVA of Squared Deviations from Group Means

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Treat	4	1.7887	0.4472	1.57	0.2033
Error	35	9.9528	0.2844		

Bartlett's Test for Homogeneity of WEIGHT Variance

Source	DF	Chi-Square	Pr > ChiSq
Treat	4	6.1708	0.1868

Dunnett's t Tests for WEIGHT

NOTE: This test controls the Type I experimentwise error for comparisons of all treatments against a control.

Alpha	0.05
Error Degrees of Freedom	35
Error Mean Square	0.41232
Critical Value of Dunnett's t	2.55790
Minimum Significant Difference	0.8212

Comparisons significant at the 0.05 level are indicated by ***.

Treat Comparison	Difference Between Means	Simultaneous 95% Confidence Limits	
25 - 0	0.5080	-0.3133	1.3292
0.1 - 0	0.4326	-0.3886	1.2539
10 - 0	0.0530	-0.7682	0.8743
1 - 0	-0.3188	-1.1401	0.5024

ANOVA FOR WEIGHT OF FROGS ACROSS TREATMENT BY SEX 133

-- Sex=M --

The ANOVA Procedure

Class Level Information

Class	Levels	Values
Treat	5	0 0.1 1 10 25

Number of observations 40

Dependent Variable: WEIGHT

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	4	1.48024068	0.37006017	1.07	0.3857
Error	35	12.09767780	0.34564794		
Corrected Total	39	13.57791848			

R-Square	Coeff Var	Root MSE	WEIGHT Mean
0.109018	18.29415	0.587918	3.213696

Source	DF	Anova SS	Mean Square	F Value	Pr > F
Treat	4	1.48024068	0.37006017	1.07	0.3857

Levene's Test for Homogeneity of WEIGHT Variance
ANOVA of Squared Deviations from Group Means

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Treat	4	0.5338	0.1334	0.35	0.8426
Error	35	13.3648	0.3819		

Bartlett's Test for Homogeneity of WEIGHT Variance

Source	DF	Chi-Square	Pr > ChiSq
Treat	4	2.9535	0.5656

Dunnett's t Tests for WEIGHT

NOTE: This test controls the Type I experimentwise error for comparisons of all treatments against a control.

Alpha	0.05
Error Degrees of Freedom	35
Error Mean Square	0.345648
Critical Value of Dunnett's t	2.55790
Minimum Significant Difference	0.7519

Comparisons significant at the 0.05 level are indicated by ***.

Treat Comparison	Difference Between Means	Simultaneous 95% Confidence Limits	
0.1 - 0	0.3171	-0.4348	1.0691
25 - 0	0.1513	-0.6006	0.9033
10 - 0	-0.0259	-0.7779	0.7260
1 - 0	-0.2599	-1.0118	0.4920

NONPARAMETRIC COMPARISON OF FROG WEIGHT ACROSS TREATMENTS BY SEX 137

-- Sex=F --

The NPAR1WAY Procedure

Wilcoxon Scores (Rank Sums) for Variable WEIGHT
Classified by Variable Treat

Treat	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
0	8	157.0	164.0	29.574764	19.6250
0.1	8	196.0	164.0	29.574764	24.5000
1	8	98.0	164.0	29.574764	12.2500
10	8	162.0	164.0	29.574764	20.2500
25	8	207.0	164.0	29.574764	25.8750

Kruskal-Wallis Test

Chi-Square	6.6604
DF	4
Pr > Chi-Square	0.1550

Median Scores (Number of Points Above Median) for Variable WEIGHT
Classified by Variable Treat

Treat	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
0	8	4.0	4.0	1.281025	0.5000
0.1	8	5.0	4.0	1.281025	0.6250
1	8	2.0	4.0	1.281025	0.2500
10	8	4.0	4.0	1.281025	0.5000
25	8	5.0	4.0	1.281025	0.6250

Median One-Way Analysis

Chi-Square	2.9250
DF	4
Pr > Chi-Square	0.5705

--- Sex=M ---

The NPAR1WAY Procedure

Wilcoxon Scores (Rank Sums) for Variable WEIGHT
Classified by Variable Treat

Treat	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
0	8	145.0	164.0	29.574764	18.1250
0.1	8	219.0	164.0	29.574764	27.3750
1	8	110.0	164.0	29.574764	13.7500
10	8	170.0	164.0	29.574764	21.2500
25	8	176.0	164.0	29.574764	22.0000

Kruskal-Wallis Test

Chi-Square	5.9287
DF	4
Pr > Chi-Square	0.2045

Median Scores (Number of Points Above Median) for Variable WEIGHT
Classified by Variable Treat

Treat	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
0	8	2.0	4.0	1.281025	0.2500
0.1	8	6.0	4.0	1.281025	0.7500
1	8	3.0	4.0	1.281025	0.3750
10	8	5.0	4.0	1.281025	0.6250
25	8	4.0	4.0	1.281025	0.5000

Median One-Way Analysis

Chi-Square	4.8750
DF	4
Pr > Chi-Square	0.3004

-- Sex=F --

The ANOVA Procedure

Class Level Information

Class	Levels	Values
Treat	5	0 0.1 1 10 25

Number of observations 40

Dependent Variable: LENGTH

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	4	0.46918445	0.11729611	4.21	0.0069
Error	35	0.97530236	0.02786578		
Corrected Total	39	1.44448681			

R-Square	Coeff Var	Root MSE	LENGTH Mean
0.324810	5.507625	0.166930	3.030898

Source	DF	Anova SS	Mean Square	F Value	Pr > F
Treat	4	0.46918445	0.11729611	4.21	0.0069

Levene's Test for Homogeneity of LENGTH Variance
ANOVA of Squared Deviations from Group Means

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Treat	4	0.00398	0.000996	1.29	0.2939
Error	35	0.0271	0.000774		

Bartlett's Test for Homogeneity of LENGTH Variance

Source	DF	Chi-Square	Pr > ChiSq
Treat	4	2.7074	0.6079

Dunnett's t Tests for LENGTH

NOTE: This test controls the Type I experimentwise error for comparisons of all treatments against a control.

Alpha	0.05
Error Degrees of Freedom	35
Error Mean Square	0.027866
Critical Value of Dunnett's t	2.55790
Minimum Significant Difference	0.2135

Comparisons significant at the 0.05 level are indicated by ***.

Treat Comparison	Difference Between Means	Simultaneous 95% Confidence Limits	
0.1 - 0	0.14893	-0.06457	0.36242
25 - 0	0.13545	-0.07804	0.34895
10 - 0	-0.04026	-0.25375	0.17324
1 - 0	-0.13709	-0.35058	0.07641

ANOVA FOR LENGTH OF FROGS ACROSS TREATMENT BY SEX 145

-- Sex=M --

The ANOVA Procedure

Class Level Information

Class	Levels	Values
Treat	5	0 0.1 1 10 25

Number of observations 40

Dependent Variable: LENGTH

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	4	0.15470697	0.03867674	1.38	0.2623
Error	35	0.98388783	0.02811108		
Corrected Total	39	1.13859480			

R-Square	Coeff Var	Root MSE	LENGTH Mean
0.135875	5.628738	0.167664	2.978707

Source	DF	Anova SS	Mean Square	F Value	Pr > F
Treat	4	0.15470697	0.03867674	1.38	0.2623

Levene's Test for Homogeneity of LENGTH Variance
ANOVA of Squared Deviations from Group Means

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Treat	4	0.00309	0.000772	0.36	0.8370
Error	35	0.0756	0.00216		

Bartlett's Test for Homogeneity of LENGTH Variance

Source	DF	Chi-Square	Pr > ChiSq
Treat	4	2.3528	0.6712

Dunnett's t Tests for LENGTH

NOTE: This test controls the Type I experimentwise error for comparisons of all treatments against a control.

Alpha	0.05
Error Degrees of Freedom	35
Error Mean Square	0.028111
Critical Value of Dunnett's t	2.55790
Minimum Significant Difference	0.2144

Comparisons significant at the 0.05 level are indicated by ***.

Treat Comparison	Difference Between Means	Simultaneous 95% Confidence Limits	
0.1 - 0	0.07055	-0.14388	0.28498
25 - 0	0.03457	-0.17986	0.24901
10 - 0	-0.03291	-0.24735	0.18152
1 - 0	-0.11130	-0.32573	0.10313

-- Sex=F --

The NPAR1WAY Procedure

Wilcoxon Scores (Rank Sums) for Variable LENGTH
Classified by Variable Treat

Treat	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
0	8	159.0	164.0	29.574764	19.8750
0.1	8	224.0	164.0	29.574764	28.0000
1	8	89.0	164.0	29.574764	11.1250
10	8	133.0	164.0	29.574764	16.6250
25	8	215.0	164.0	29.574764	26.8750

Kruskal-Wallis Test

Chi-Square	11.7183
DF	4
Pr > Chi-Square	0.0196

Median Scores (Number of Points Above Median) for Variable LENGTH
Classified by Variable Treat

Treat	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
0	8	3.0	4.0	1.281025	0.3750
0.1	8	6.0	4.0	1.281025	0.7500
1	8	2.0	4.0	1.281025	0.2500
10	8	3.0	4.0	1.281025	0.3750
25	8	6.0	4.0	1.281025	0.7500

Median One-Way Analysis

Chi-Square	6.8250
DF	4
Pr > Chi-Square	0.1454

NONPARAMETRIC COMPARISON OF FROG LENGTHS ACROSS TREATMENTS BY SEX 151

-- Sex=M --

The NPAR1WAY Procedure

Wilcoxon Scores (Rank Sums) for Variable LENGTH
Classified by Variable Treat

Treat	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
0	8	154.0	164.0	29.574764	19.2500
0.1	8	217.0	164.0	29.574764	27.1250
1	8	106.0	164.0	29.574764	13.2500
10	8	162.0	164.0	29.574764	20.2500
25	8	181.0	164.0	29.574764	22.6250

Kruskal-Wallis Test

Chi-Square	6.0055
DF	4
Pr > Chi-Square	0.1987

The NPAR1WAY Procedure

Median Scores (Number of Points Above Median) for Variable LENGTH
Classified by Variable Treat

Treat	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
0	8	3.0	4.0	1.281025	0.3750
0.1	8	6.0	4.0	1.281025	0.7500
1	8	3.0	4.0	1.281025	0.3750
10	8	4.0	4.0	1.281025	0.5000
25	8	4.0	4.0	1.281025	0.5000

Median One-Way Analysis

Chi-Square	2.9250
DF	4
Pr > Chi-Square	0.5705

ANOVA FOR WEIGHT OF FROGS ACROSS POSITIVE CONTROLS BY SEX 153

--- Sex=F ---

The ANOVA Procedure

Class Level Information

Class	Levels	Values
Treat	3	DHT E2 ETOH

Number of observations 24

Dependent Variable: WEIGHT

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	2	3.17425911	1.58712955	0.86	0.4380
Error	21	38.80382271	1.84780108		
Corrected Total	23	41.97808182			

R-Square	Coeff Var	Root MSE	WEIGHT Mean
0.075617	39.27037	1.359338	3.461487

Source	DF	Anova SS	Mean Square	F Value	Pr > F
Treat	2	3.17425911	1.58712955	0.86	0.4380

Levene's Test for Homogeneity of WEIGHT Variance
ANOVA of Squared Deviations from Group Means

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Treat	2	111.3	55.6423	1.88	0.1779
Error	21	622.7	29.6509		

Bartlett's Test for Homogeneity of WEIGHT Variance

Source	DF	Chi-Square	Pr > ChiSq
Treat	2	32.4133	<.0001

Dunnett's t Tests for WEIGHT

NOTE: This test controls the Type I experimentwise error for comparisons of all treatments against a control.

Alpha	0.05
Error Degrees of Freedom	21
Error Mean Square	1.847801
Critical Value of Dunnett's t	2.37033
Minimum Significant Difference	1.611

Comparisons significant at the 0.05 level are indicated by ***.

Treat Comparison	Difference Between Means	Simultaneous 95% Confidence Limits	
ETOH - DHT	-0.5422	-2.1532	1.0688
E2 - DHT	-0.8832	-2.4943	0.7278

-- Sex=M --

The ANOVA Procedure

Class Level Information

Class	Levels	Values
Treat	3	DHT E2 ETOH

Number of observations 24

Dependent Variable: WEIGHT

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	2	4.32184582	2.16092291	1.94	0.1680
Error	21	23.33843834	1.11135421		
Corrected Total	23	27.66028416			

R-Square	Coeff Var	Root MSE	WEIGHT Mean
0.156247	32.05405	1.054208	3.288845

Source	DF	Anova SS	Mean Square	F Value	Pr > F
Treat	2	4.32184582	2.16092291	1.94	0.1680

Levene's Test for Homogeneity of WEIGHT Variance
ANOVA of Squared Deviations from Group Means

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Treat	2	32.0265	16.0133	4.05	0.0327
Error	21	83.1297	3.9586		

Bartlett's Test for Homogeneity of WEIGHT Variance

Source	DF	Chi-Square	Pr > ChiSq
Treat	2	20.9573	<.0001

Dunnett's t Tests for WEIGHT

NOTE: This test controls the Type I experimentwise error for comparisons of all treatments against a control.

Alpha	0.05
Error Degrees of Freedom	21
Error Mean Square	1.111354
Critical Value of Dunnett's t	2.37033
Minimum Significant Difference	1.2494

Comparisons significant at the 0.05 level are indicated by ***.

Treat Comparison	Difference Between Means	Simultaneous 95% Confidence Limits	
ETOH - DHT	-0.6254	-1.8748	0.6240
E2 - DHT	-1.0317	-2.2811	0.2177

--- Sex=F ---

The NPAR1WAY Procedure

Wilcoxon Scores (Rank Sums) for Variable WEIGHT
Classified by Variable Treat

Treat	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
DHT	8	91.0	100.0	16.329932	11.3750
E2	8	85.0	100.0	16.329932	10.6250
ETOH	8	124.0	100.0	16.329932	15.5000

Kruskal-Wallis Test

Chi-Square	2.2050
DF	2
Pr > Chi-Square	0.3320

Median Scores (Number of Points Above Median) for Variable WEIGHT
Classified by Variable Treat

Treat	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
DHT	8	4.0	4.0	1.179536	0.5000
E2	8	3.0	4.0	1.179536	0.3750
ETOH	8	5.0	4.0	1.179536	0.6250

Median One-Way Analysis

Chi-Square	0.9583
DF	2
Pr > Chi-Square	0.6193

--- Sex=M ---

The NPAR1WAY Procedure

Wilcoxon Scores (Rank Sums) for Variable WEIGHT
Classified by Variable Treat

Treat	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
DHT	8	115.0	100.0	16.329932	14.3750
E2	8	69.0	100.0	16.329932	8.6250
ETOH	8	116.0	100.0	16.329932	14.5000

Kruskal-Wallis Test

Chi-Square	3.6050
DF	2
Pr > Chi-Square	0.1649

Median Scores (Number of Points Above Median) for Variable WEIGHT
Classified by Variable Treat

Treat	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
DHT	8	5.0	4.0	1.179536	0.6250
E2	8	2.0	4.0	1.179536	0.2500
ETOH	8	5.0	4.0	1.179536	0.6250

Median One-Way Analysis

Chi-Square	2.8750
DF	2
Pr > Chi-Square	0.2375

--- Sex=F ---

The ANOVA Procedure

Class Level Information

Class	Levels	Values
Treat	3	DHT E2 ETOH

Number of observations 24

Dependent Variable: LENGTH

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	2	0.02901222	0.01450611	0.16	0.8568
Error	21	1.95623777	0.09315418		
Corrected Total	23	1.98524999			

R-Square	Coeff Var	Root MSE	LENGTH Mean
0.014614	9.974886	0.305212	3.059801

Source	DF	Anova SS	Mean Square	F Value	Pr > F
Treat	2	0.02901222	0.01450611	0.16	0.8568

Levene's Test for Homogeneity of LENGTH Variance
ANOVA of Squared Deviations from Group Means

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Treat	2	0.2482	0.1241	2.06	0.1530
Error	21	1.2675	0.0604		

Bartlett's Test for Homogeneity of LENGTH Variance

Source	DF	Chi-Square	Pr > ChiSq
Treat	2	21.9346	<.0001

Dunnett's t Tests for LENGTH

NOTE: This test controls the Type I experimentwise error for comparisons of all treatments against a control.

Alpha	0.05
Error Degrees of Freedom	21
Error Mean Square	0.093154
Critical Value of Dunnett's t	2.37033
Minimum Significant Difference	0.3617

Comparisons significant at the 0.05 level are indicated by ***.

Treat Comparison	Difference Between Means	Simultaneous 95% Confidence Limits	
ETOH - DHT	-0.06883	-0.43056	0.29290
E2 - DHT	-0.07785	-0.43958	0.28388

--- Sex=M ---

The ANOVA Procedure

Class Level Information

Class	Levels	Values
Treat	3	DHT E2 ETOH

Number of observations 24

Dependent Variable: LENGTH

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	2	0.15041338	0.07520669	0.91	0.4167
Error	21	1.72987563	0.08237503		
Corrected Total	23	1.88028900			

R-Square	Coeff Var	Root MSE	LENGTH Mean
0.079995	9.484117	0.287011	3.026223

Source	DF	Anova SS	Mean Square	F Value	Pr > F
Treat	2	0.15041338	0.07520669	0.91	0.4167

Levene's Test for Homogeneity of LENGTH Variance
ANOVA of Squared Deviations from Group Means

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Treat	2	0.1367	0.0684	3.85	0.0375
Error	21	0.3725	0.0177		

Bartlett's Test for Homogeneity of LENGTH Variance

Source	DF	Chi-Square	Pr > ChiSq
Treat	2	13.2247	0.0013

Dunnett's t Tests for LENGTH

NOTE: This test controls the Type I experimentwise error for comparisons of all treatments against a control.

Alpha	0.05
Error Degrees of Freedom	21
Error Mean Square	0.082375
Critical Value of Dunnett's t	2.37033
Minimum Significant Difference	0.3402

Comparisons significant at the 0.05 level are indicated by ***.

Treat Comparison	Difference Between Means	Simultaneous 95% Confidence Limits	
ETOH - DHT	-0.1378	-0.4780	0.2023
E2 - DHT	-0.1871	-0.5272	0.1531

NONPARAMETRIC COMPARISON OF FROG LENGTHS ACROSS POSITIVE CONTROLS BY SEX 173

--- Sex=F ---

The NPAR1WAY Procedure

Wilcoxon Scores (Rank Sums) for Variable LENGTH
Classified by Variable Treat

Treat	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
DHT	8	78.0	100.0	16.329932	9.750
E2	8	110.0	100.0	16.329932	13.750
ETOH	8	112.0	100.0	16.329932	14.000

Kruskal-Wallis Test

Chi-Square	1.8200
DF	2
Pr > Chi-Square	0.4025

The NPAR1WAY Procedure

Median Scores (Number of Points Above Median) for Variable LENGTH
Classified by Variable Treat

Treat	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
DHT	8	2.0	4.0	1.179536	0.2500
E2	8	5.0	4.0	1.179536	0.6250
ETOH	8	5.0	4.0	1.179536	0.6250

Median One-Way Analysis

Chi-Square	2.8750
DF	2
Pr > Chi-Square	0.2375

NONPARAMETRIC COMPARISON OF FROG LENGTHS ACROSS POSITIVE CONTROLS BY SEX 175

--- Sex=M ---

The NPAR1WAY Procedure

Wilcoxon Scores (Rank Sums) for Variable LENGTH
Classified by Variable Treat

Treat	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
DHT	8	103.0	100.0	16.329932	12.8750
E2	8	92.0	100.0	16.329932	11.5000
ETOH	8	105.0	100.0	16.329932	13.1250

Kruskal-Wallis Test

Chi-Square	0.2450
DF	2
Pr > Chi-Square	0.8847

The NPAR1WAY Procedure

Median Scores (Number of Points Above Median) for Variable LENGTH
Classified by Variable Treat

Treat	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
DHT	8	4.0	4.0	1.179536	0.50
E2	8	4.0	4.0	1.179536	0.50
ETOH	8	4.0	4.0	1.179536	0.50

Median One-Way Analysis

Chi-Square	0.0000
DF	2
Pr > Chi-Square	1.0000

```
                         AVERAGE LENGTH OF FROGS OVER TREATMENTS BY SEX                           177

------------------------------------------- Sex=F ---------------------------------------------

                          Plot of LENGTH*CONC.  Symbol used is '*'.

   LENGTH é
          é
     3.15 ê
          é
          é
          é  *
          é
          é                                                                     *
     3.10 ê
          é
          é
          é
          é
          é
     3.05 ê
          é
          é
          é
          é
          é  *
     3.00 ê
          é
          é
          é
          é
          é                           *
     2.95 ê
          é
          é
          é
          é
          é
     2.90 ê
          é
          é
          é
          é
          é     *
     2.85 ê
          é
          èâââââââââââââââââââââââââââââââââââââââââââââââââââââââââââââââââââââââââââââââââââââ
             0             5            10            15            20            25

                                              CONC
```

```
                          AVERAGE LENGTH OF FROGS OVER TREATMENTS BY SEX                         178

------------------------------------------- Sex=M --------------------------------------------

                             Plot of LENGTH*CONC.  Symbol used is '*'.

LENGTH é
 3.050 ê
       é
       é
       é  *
       é
 3.025 ê
       é
       é
       é
       é
 3.000 ê
       é
       é
       é
       é                                                                         *
 2.975 ê
       é
       é
       é
       é
 2.950 ê  *
       é                              *
       é
       é
       é
 2.925 ê
       é
       é
       é
       é
 2.900 ê
       é
       é
       é
       é
 2.875 ê
       é
       é
       é     *
       é
 2.850 ê
       èââêâââââââââââââêâââââââââââââêâââââââââââââêâââââââââââââêâââââââââââââêââ
          0             5            10            15            20            25

                                            CONC
```

```
------------------------------------------- Sex=F ------------------------------------------

                        Plot of WEIGHT*CONC.  Symbol used is '*'.

WEIGHT é
       é
   3.7 ê
       é
       é
       é                                                                        *
   3.6 ê
       é
       é
       é
   3.5 ê
       é
       é
       é
   3.4 ê  *
       é
       é
       é
   3.3 ê
       é
       é                              *
       é  *
   3.2 ê
       é
       é
       é
   3.1 ê
       é
       é
       é
   3.0 ê
       é
       é
       é
   2.9 ê
       é
       é     *
       é
   2.8 ê
       é
       èââêâââââââââââââêâââââââââââââêâââââââââââââêâââââââââââââêâââââââââââââêââ
          0             5            10            15            20            25

                                            CONC
```

Data Evaluation Report on Response of *Xenopus laevis* to Atrazine Exposure: Assessment of the Mechanism of Action of Atrazine.

EPA MRID Number 458677-04

```
                         AVERAGE WEIGHT OF FROGS OVER TREATMENTS BY SEX                         180

-------------------------------------------- Sex=M --------------------------------------------

                          Plot of WEIGHT*CONC.  Symbol used is '*'.

      WEIGHT é
             é
         3.4 ê  *
             é
             é
             é
             é
             é
         3.3 ê
             é
             é
             é
             é
             é
         3.2 ê
             é
             é                                                                        *
             é
             é
             é                             *
         3.1 ê
             é
             é
             é  *
             é
             é
         3.0 ê
             é
             é
             é
             é
             é
         2.9 ê
             é
             é     *
             é
             é
             é
         2.8 ê
             é
             èââêâââââââââââââêâââââââââââââêâââââââââââââêâââââââââââââêâââââââââââââêââ
                0             5             10            15            20            25

                                             CONC
```

Data Evaluation Report on Histology of the Gonads and Analysis of Hormone Levels in the Native Bullfrog (*Rana catesbiena*) Collected from Agricultural Areas in Southern Iowa: Pilot Project.

EPA MRID Number 458677-05

Data Requirement:	EPA DP Barcode	D288775
	EPA MRID	458677-05
	EPA Guideline	70-1(Special Study)

Test material: Purity: not reported

Common name: Atrazine
Chemical name: IUPAC
CAS name 6-chloro-N-ethyl-N'-(1-methylethyl)-1,3,5-triazine-2,4-diamine
CAS No. 1912-24-9
Synonyms
EPA PC Code: 80803

Thomas M Steeger

Primary Reviewer: Thomas M. Steeger, Ph.D., Senior Biologist **Date:** March 27, 2003
Environmental Fate and Effects Division, ERB 4,
U. S. Environmental Protection Agency

Joseph E. Tietge

Secondary Reviewer(s): Joseph E. Tietge, M.S., Research Aquatic Biologist **Date:** April 16, 2003
Mid-Continent Ecology Division,
National Health and Environmental Effects Research Laboratory (Duluth),
U. S. Environmental Protection Agency

Stephanie R. Irene, 5/6/03

Stephanie Irene, Ph.D., Senior Advisor **Date:**
Environmental Fate and Effects Division, ERB 3,
U. S. Environmental Protection Agency

Mary J. Frankenberry

Mary J. Frankenberry, Senior Statistician **Date:** 5/5/03
Environmental Fate and Effects Division, ERB 3,
U. S. Environmental Protection Agency

EPA PC Code 080803

Date Evaluation Completed: 06/01/2003

CITATION: Crabtree, C.; E. E. Smith; J. A. Carr. 2003. Histology of the gonads and analysis of hormone levels in the native bull frog (*Rana catesbiena*) collected from agricultural areas in southern Iowa: pilot project. The Institute of Environmental and Human Health, Texas Technical University, Lubbock, Texas. Sponsor: Syngenta Crop Protection, Inc. Laboratory Identification Number ECORISK Number TTU-02.

EXECUTIVE SUMMARY:

This study presents the results of Phase 1 of a three-phase study where 14 pond sites in southern Iowa (3 reference and 11 atrazine-exposed) were characterized. Experimental sites were located in corn and soybean-dominated agriculture areas. Pond sizes ranged from 0.14 ha to 2.94 ha with average watershed areas ranging from 2.19 ha to 84.02 ha. Atrazine concentrations in reference ponds averaged 0.06 µg/L. Mean atrazine concentrations in corn-dominated sites over the June to September ranged from 1.07 to 19.26 µg/L; atrazine was highest in corn-dominated ponds in June/July with a maximum value of 35.07 µg/L. For soybean-dominated watersheds, the highest residues ranged from 3.19 to 3.85 µg/L. Similarly, maximum deisopropyl atrazine residue concentrations were highest in corn-dominated areas in June/July at 4.17 µg/L. Maximum desethyl atrazine (DEA) residues were highest in corn-dominated ponds at 16.55 µg/L in June/July and 16.10 µg/L in August. Residues of diaminochlortriazine (DACT) remained relatively constant during the sampling period and once again, the highest average residues (0.65 µg/L) were from corn-dominated ponds.

Bullfrogs (*Rana catesbiena*) were present at all sites in sufficient numbers for collection; however, not all life stages were collected at every site.. No significant differences were found for adult body weight or snout-vent length (SVL). Mean weight and SVL for juvenile females were significantly lower in reference sites than atrazine-exposure sites. Mean SVL for juvenile males was significantly lower in reference sites than in atrazine-exposure sites; however mean weight of juvenile males was not statistically different between sites. Gonadal somatic index (GSI) was not significantly different between sites for either adults or juveniles. No gross gonadal abnormalities were observed based on visual examination (gross morphology). The incidence of external abnormalities was less than 1% of the total frogs caught.

The number of water samples may have been insufficient to characterize the exposure potential to atrazine, particularly in reference sites. While an effort was made to characterize a limited number of herbicides, no effort appears to have been made to characterize other pesticides in general. Based on the preliminary results, none of the indices measured (weight, length, GSI or the incidence of gross gonadal deformities) in the bullfrog indicate that variable exposure levels to atrazine and other triazine degradates are adversely affecting this species. While the bullfrog appears to be clearly present, the relevancy of this species is questionable because there isn't much information available on the bullfrog relative to some of the indices of interest, *i.e.*, steroid hormone levels, aromatase levels, background incidence, and types of gonadal abnormalities.

I. MATERIALS AND METHODS

GUIDELINE FOLLOWED: Nonguideline Study

COMPLIANCE: Not conducted under full GLP; however, most practices as defined by 40 CFR Part 160, August 19, 1989 were established for this study, including but not limited to:

- Written, authorized preliminary protocol
- Written, authorized Standard Operating Procedures for all key procedures.
- Organization and Personnel were sufficient in terms of number, education, training and experience.
- Facilities were of suitable size and construction
- Equipment used was of appropriate design and adequate capacity.
- Independent QA Inspection was conducted of raw data..
- Interim Report was written
- Raw data, documentation, records, protocols, and final report will be archived.

A. MATERIALS:

1. Test Material Atrazine

Description: Not reported

Lot No./Batch No. : Not reported

Purity: NA
Stability of compound under test conditions: Not reported

Storage conditions of test chemicals:_ Not reported

2. Test organism:

Species: Native bull frog (*Rana catesbiena*)

Age at test initiation:
Weight at study initiation: (mean and range) Not reported
Length at study initiation: (mean and range) Not reported

Source: Field-collected at Edith Angel Environmental Center,
The Institute of Environmental and Human Health
Texas Tech University
44351 State Hwy 13
Chariton, Iowa 50049

B. STUDY DESIGN:

Objective:

1. To select sites and validate biochemical, analytical methods and sampling techniques to assess the effects of atrazine on kidney and gonad histology of bullfrogs (*Rana catesbiena*) and other species collected from various field sites in southern Iowa.
2. Develop biochemical and analytical methods for determination of blood steroid hormone levels and gonadal aromatase activity.
3. Conduct histological analysis of the gonads and kidneys of collected frogs.
4. Calculate the gonadal somatic index (GSI) of collected frogs.
5. Measure atrazine levels in the aquatic environments where frogs are collected.
6. Describe the study sites, physically, biologically, and chemically, by recording the following data for each site: shape/area, depth, plant life, approximate watershed area, major crops in the watershed, pesticides used on the major crops, and water quality indices (temperature, pH, dissolved oxygen, and conductivity).
7. Describe the morphology of collected frogs by recording the sex, weight, snout-vent length (SVL), and any physical abnormalities observed.

1. Experimental Conditions

Sampling was conducted in mid-summer (June/July 2002), late-summer (August 2002), Fall (September 2002) and Spring 2003 (no data reported as of yet) in Phase I of a three- phase study. Each pond/lake was sampled once during each sampling period. Frogs at all life stages (tadpole through adult) were collected at each sampling.

Corn/soybean rotational cropping patterns in 11 atrazine-use areas (K-1, R1 - R4, R6, S1 - S3, T1, W1) and 3 reference sites (isolated from corn/soybean culture) (F1, M1, and P1) were evaluated. Reference site watersheds were either primary hardwood forest (M1) or turf (P1, F1). Experimental site watersheds were a combination of corn, soybean and pasture/hay. Experimental sites R1, R2, R3, S2, T1 and W1 were planted in soybeans and treated primarily with glyphosate in 2002. Ponds K1, R4, R6, S1 and S3 were planted in corn and treated with atrazine in combination with one or more of the following herbicides: acetochlor, s-metolachlor, nicosulfuron, rimsulfuron or 2,4-D.

Bifenthrin was the only insecticide applied to corn. Most of the soybeans were genetically modified oganisms engineered for resistance to glyphosate. Soybeans were treated in spring with Roundup®. A variety of herbicides were used in the watersheds of corn (atrazine, acetochlor, s-metolachlor, clomazone, ethalfluralin, nicosulfuron, rimsulfuron and 2,4-D.

At each sampling, duplicate water samples were collected from each study site; two 250-mL samples were collected from 4 sites evenly distributed around each water body (N, S, E and W) and collected at a depth of 10 cm. Each set of four 250-mL samples were pooled into a 1-L composite. At the time of collection, temperature, DO, pH and conductivity were measured. One sediment sample was collected from each site during late-ummer sampling.

Samples were analyzed for atrazine and its metabolites, simizine and metolochlor.

Frogs were collected by dip net for one hour at night. All frogs that could be collected within the 1-hour period were held in buckets of pond water until the sampling period ended. All frogs with physical abnormalities were immediately selected for analysis and the remainder were randomly selected from the pooled sample until a sample size of 10 (15 during early fall) was achieved. Tadpoles were collected using dip-nets just prior to or concurrent with frog sampling until a total of 10 tadpoles were collected. Remaining frogs were released back into the pond.

Blood samples were collected from adults by cardiac puncture. Species, sex, weight and SVL for each necropsied specimen was recorded (apparently after each of the animals had been bled and necropsied). During early fall sampling, right gonad was extracted from 5 of the 15 collected animals, frozen in liquid nitrogen for use in ^3H_2O-release aromatase assay. The remainder of the carcass was fixed in Bouin's. A random sample of 340 male and female frogs (73% of all the frogs sampled in 2002) were examined visually.

II. <u>RESULTS</u> and <u>DISCUSSION</u>: [All results discussed in this section and the next are those reported by the study authors. Although supplemental data are typically used in a qualitative manner only, EFED verified spreadsheet data and ran basic statistical analyses on the major study parameters. See attached appendix. If results appeared to differ in any substantive way, the difference was reported in the text below.]

Pond sizes ranged from 0.14 ha to 2.94 ha. The average watershed areas ranged from 2.19 ha to 84.02 ha. Dissolved oxygen in terms of percent saturation was surprisingly low during the late-summer and fall samplings dropping as low as 1.9% saturation. In pond T1, pH is reported to have dropped to 1.31 and is likely a typo.

Although atrazine concentrations in corn-dominated sites over the June to September sampling period averaged 5.52 µg/L (Table 1), the means from each of the ponds ranged from 1.07 to 19.26 µg/L. Atrazine was highest in corn-dominated ponds in June/July with the maximum value of 35.07 µg/L; experimental pond R6 (corn) exhibited the highest atrazine levels ranging from 5.7 to 35.07 µg/L. For soybean-dominated watersheds, pond W1 had the highest residues ranging from 3.19 to 3.85 µg/L. Similarly, deisopropyl atrazine concentrations were highest in corn-dominated areas and averaged 0.89 µg/L; however maximum residues were recorded in June/July at 4.17 µg/L. Desethyl atrazine (DEA) residues were highest in corn-dominated ponds; however residues from June through August were relatively consistent. Maximum residues for DEA were at 16.55 µg/L in June/July and 16.10 µg/L in August. Residues of diaminochlortriazine (DACT) remained relatively constant during the sampling period and once again, the highest average residues (0.65 µg/L) were from corn-dominated ponds. The maximum residue detected (2.33 µg/L) occurred twice, once in June/July and again in September.

Metolachlor residues averaged between 0.05 and 0.06 µg/L across all sample sites while simazine residues averaged 0.05 µg/L. The detection limits for both metolachlor and simazine are 0.1 µg/L and thus residues were at or below detection limits.

Table 1. Mean atrazine, diaminochlorotriazine (DACT), deisopropyl atrazine, and desethyl atrazine residues in pond water collected at refernece[a], corn-dominated[b] and soybean-dominated[c] sites.

Chemical Residues	Reference µg/L	Corn µg/L	Soybean µg/L
Atrazine	0.06	5.52	1.05
Diaminochlorotriazine	0.10	0.65	0.24
Deisopropyl atrazine	0.05	0.89	0.21
Desethyl atrazine	0.05	3.18	0.65

[a] reference site include ponds M1, P1 and F1
[b] corn-dominated sites include ponds R4, R6, K1, S1 and S3
[c] soybean-dominated sites include ponds R1, R2, R3, S2, T1 and W1

Frogs without tails but less than 70 g were classified as juveniles, while frogs without tails but weighing ≥ 70 g were classified as adults. More females than males were collected in sampling period 1 (50% vs 35%) and in period 2 (54% vs 40%); however, in sampling period 3, the percentage of males and females was roughly similar 50% vs 49%). Overall, for the three ponds combined, females outnumbered males (51% vs 43%) and juveniles outnumbered the adults (52% vs 44%). Species other than bullfrogs represented 6% of all collected frogs.

T-test comparisons between reference sites and experimental sites for adult males and adult females resulted in no significant difference for either weight or length (**Table 2**). In general there was a trend for male and female adults to weigh more at reference sites. However, mean weight and SVL for juvenile females was significantly lower in reference sites than experimental sites. There were no differences in the gonadosomatic index for males (weight of the right gonad ÷ frog's weight) or females collected at reference versus experimental sites (**Table 3**).

No gross morphological abnormalities were observed (n = 340) in either male or female gonads collected across reference and experimental sites.

Table 4 summarizes the total number of bullfrogs caught and sampled in reference and experimental sites and the sexes of those animals over the three sampling periods. This information was taken from Tables 10 through 12 of the report. Based on these data, the percentage of males in reference samples ranged from 11 to 56% over the three sampling periods, while the percentage of males in experimental site samples ranged from 41 to 49%. In reference sites, the percentage of frogs where the sex could not be determined based on gross morphology ranged from 0 to 22% while in experimental sites, the percentage ranged form 1.4% to 5.9%.

Table 2. Necropsied carcass weight and snout-to-vent length (SVL) for male and female, adult and juvenile bullfrogs collected from reference and experimental sites.

Site Type	Sex	Stage	Weight			SVL		
			N	Mean	SD	N	Mean	SD
Reference	male	adult	14	147.33	47.79	14	119.1	11.9
	female		16	195.61	104.58	15	126.6	20.7
	male	juvenile	22	18.65	19.03	22	55.5	18.8
	female		32	15.63	13.13	32	53.7	15.5
Experimental	male	adult	78	134.40	57.74	78	114.9	15.9
	female		68	160.23	98.84	65	117.7	17.9
	male	juvenile	78	26.56	17.58	78	65.6	15.3
	female		103	26.99	16.75	103	65.6	14.5

Table 3. Gonadosomatic index for bullfrogs collected during sampling period 3

Site Type	Sex	Stage	N	Mean	Standard Deviation
Reference	male	Adult	6	0.07%	0.01%
	female		3	1.01%	0.52%
	male	Juvenile	2	0.05%	0.02%
	female		3	0.67%	0.16%
Experimental	male	Adult	15	0.06%	0.02%
	female		15	0.93%	0.74%
	male	Juvenile	10	0.03%	0.02%
	female		15	0.60%	0.29%

Table 4. Total number of bullfrogs caught and sampled and their respective sex based on gross morphology by collection period.

Sampling Period	Treatment	Number Caught	Number Sampled	Males	Females	Unknown
June - July	Reference	18	18	2	12	4
	Experimental	394	99	41	47	11
August	Reference	42	27	9	16	2
	Experimental	447	101	44	51	6
September	Reference	87	45	25	20	0
	Experimental	286	146*	71	75	2

* Pond R3 and W1 reported as 14 frogs captured but in both 15 were reported sexed.

REVIEWER'S COMMENTS:

This study represents a basic survey of a proposed study area. The limited number of water samples may not have provided sufficient characterization of the exposure potential to atrazine, particularly in reference sites. While an effort was made to characterize a limited number of herbicides, other pesticides apparently were not characterized. While the bullfrog appears to be clearly present, the relevancy of this species is questionable because no other studies looking at gonadal abnormalities are available on this species.

Weighing and measuring animals after cardiac puncture may influence the accuracy of these numbers. It is unclear why no mention is made of the number of adult animals where the sex could not be determined; in reference ponds the percentage was as high as 22%. Because the histology data have not been compiled, it is premature to conclude anything about the incidence of intersex in the bullfrog. Initial results, though, suggest that the field-collected bullfrog is not sensitive to atrazine.

CONCLUSIONS:

This is an interim report; however, based on the preliminary results, none of the indices measured (weight, length, GSI or the incidence of gross gonadal deformities) in the bullfrog indicate that variable exposure levels to atrazine and other triazine degradates are adversely affecting this species. While the bullfrog appears to be clearly present, the relevancy of this species is questionable because there isn't much information available on the bullfrog relative to some of the indices of interest, *i.e.*, steroid hormone levels, aromatase levels, background incidence, and types of gonadal abnormalities. Additional information is needed from the final report before a definitive conclusion can be reached for this study.

AVERAGE WEIGHT OF BULLFROGS BY TYPE (REFERENCE VS EXPERIMENTAL), SEX (MALE VS FEMALE) AND STAGE (ADULT VS JUVENILE)

Obs	TYPE	SEX	STAGE	_TYPE_	_FREQ_	WEIGHT	STD	CV
1	EXP	F	A	0	171	79.9756	91.079	113.884
2	EXP	M	A	0	156	80.4827	68.817	85.505
3	EXP	UNK	A	0	12	18.5758	15.421	83.015
4	REF	F	A	0	48	75.6256	104.667	138.402
5	REF	M	A	0	36	68.6925	71.508	104.099
6	REF	UNK	A	0	3	38.8833	57.196	147.097

AVERAGE LENGTH OF BULLFROGS BY TYPE (REFERENCE VS EXPERIMENTAL), SEX (MALE VS FEMALE) AND STAGE (ADULT VS JUVENILE)

Obs	TYPE	SEX	STAGE	_TYPE_	_FREQ_	LENGTH	STD	CV
1	EXP	F	A	0	171	85.7607	30.0012	34.9824
2	EXP	M	A	0	156	90.2558	29.2040	32.3569
3	EXP	UNK	A	0	12	57.2833	13.8312	24.1453
4	REF	F	A	0	48	76.9404	38.3845	49.8886
5	REF	M	A	0	36	80.2306	35.3826	44.1011
6	REF	UNK	A	0	3	63.6333	41.8914	65.8326

NONPARAMETRIC COMPARISON OF BULLFROG WEIGHT BETWEEN REFERENCE AND EXPERIMENTAL SITES 1418

-- SEX=F STAGE=A --

The NPAR1WAY Procedure

Wilcoxon Scores (Rank Sums) for Variable WEIGHT
Classified by Variable TYPE

TYPE	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
EXP	171	19714.50	18810.0	387.917074	115.289474
REF	48	4375.50	5280.0	387.917074	91.156250

Average scores were used for ties.

Wilcoxon Two-Sample Test

Statistic	4375.5000
Normal Approximation	
Z	-2.3304
One-Sided Pr < Z	0.0099
Two-Sided Pr > |Z|	0.0198
t Approximation	
One-Sided Pr < Z	0.0103
Two-Sided Pr > |Z|	0.0207

Z includes a continuity correction of 0.5.

Kruskal-Wallis Test

Chi-Square	5.4367
DF	1
Pr > Chi-Square	0.0197

Median Scores (Number of Points Above Median) for Variable WEIGHT
Classified by Variable TYPE

TYPE	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
EXP	171	90.0	85.109589	3.068004	0.526316
REF	48	19.0	23.890411	3.068004	0.395833

Average scores were used for ties.

Median Two-Sample Test

Statistic	19.0000
Z	-1.5940
One-Sided Pr < Z	0.0555
Two-Sided Pr > |Z|	0.1109

Median One-Way Analysis

Chi-Square	2.5408
DF	1
Pr > Chi-Square	0.1109

NONPARAMETRIC COMPARISON OF BULLFROG WEIGHT BETWEEN REFERENCE AND EXPERIMENTAL SITES 1420

-- SEX=M STAGE=A --

The NPAR1WAY Procedure

Wilcoxon Scores (Rank Sums) for Variable WEIGHT
Classified by Variable TYPE

TYPE	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
EXP	156	15586.0	15054.0	300.539133	99.910256
REF	36	2942.0	3474.0	300.539133	81.722222

Average scores were used for ties.

Wilcoxon Two-Sample Test

Statistic	2942.0000
Normal Approximation	
Z	-1.7685
One-Sided Pr < Z	0.0385
Two-Sided Pr > |Z|	0.0770
t Approximation	
One-Sided Pr < Z	0.0393
Two-Sided Pr > |Z|	0.0786

Z includes a continuity correction of 0.5.

Kruskal-Wallis Test

Chi-Square	3.1334
DF	1
Pr > Chi-Square	0.0767

Median Scores (Number of Points Above Median) for Variable WEIGHT
Classified by Variable TYPE

TYPE	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
EXP	156	82.0	78.0	2.711233	0.525641
REF	36	14.0	18.0	2.711233	0.388889

Average scores were used for ties.

Median Two-Sample Test

Statistic	14.0000
Z	-1.4753
One-Sided Pr < Z	0.0701
Two-Sided Pr > |Z|	0.1401

Median One-Way Analysis

Chi-Square	2.1766
DF	1
Pr > Chi-Square	0.1401

NONPARAMETRIC COMPARISON OF BULLFROG WEIGHT BETWEEN REFERENCE AND EXPERIMENTAL SITES 1422

-------------------------------------- SEX=UNK STAGE=A --------------------------------------

The NPAR1WAY Procedure

Wilcoxon Scores (Rank Sums) for Variable WEIGHT
Classified by Variable TYPE

TYPE	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
EXP	12	102.0	96.0	6.922015	8.50
REF	3	18.0	24.0	6.922015	6.00

Average scores were used for ties.

Wilcoxon Two-Sample Test

Statistic	18.0000
Normal Approximation	
Z	-0.7946
One-Sided Pr < Z	0.2134
Two-Sided Pr > |Z|	0.4269
t Approximation	
One-Sided Pr < Z	0.2201
Two-Sided Pr > |Z|	0.4401

Z includes a continuity correction of 0.5.

Kruskal-Wallis Test

Chi-Square	0.7513
DF	1
Pr > Chi-Square	0.3861

Median Scores (Number of Points Above Median) for Variable WEIGHT
Classified by Variable TYPE

TYPE	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
EXP	12	6.0	5.60	0.80	0.500000
REF	3	1.0	1.40	0.80	0.333333

Average scores were used for ties.

Median Two-Sample Test

Statistic	1.0000
Z	-0.5000
One-Sided Pr < Z	0.3085
Two-Sided Pr > |Z|	0.6171

Median One-Way Analysis

Chi-Square	0.2500
DF	1
Pr > Chi-Square	0.6171

NONPARAMETRIC COMPARISON OF BULLFROG LENGTH BETWEEN REFERENCE AND EXPERIMENTAL SITES 1424

--------------------------------------- SEX=F STAGE=A ---------------------------------------

The NPAR1WAY Procedure

Wilcoxon Scores (Rank Sums) for Variable SVL
Classified by Variable TYPE

TYPE	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
EXP	168	18998.0	18144.0	376.994577	113.083333
REF	47	4222.0	5076.0	376.994577	89.829787

Average scores were used for ties.

Wilcoxon Two-Sample Test

Statistic	4222.0000
Normal Approximation	
Z	-2.2640
One-Sided Pr < Z	0.0118
Two-Sided Pr > |Z|	0.0236
t Approximation	
One-Sided Pr < Z	0.0123
Two-Sided Pr > |Z|	0.0246

Z includes a continuity correction of 0.5.

Kruskal-Wallis Test

Chi-Square	5.1315
DF	1
Pr > Chi-Square	0.0235

Median Scores (Number of Points Above Median) for Variable SVL
Classified by Variable TYPE

TYPE	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
EXP	168	89.0	83.609302	3.037120	0.529762
REF	47	18.0	23.390698	3.037120	0.382979

Average scores were used for ties.

Median Two-Sample Test

Statistic	18.0000
Z	-1.7749
One-Sided Pr < Z	0.0380
Two-Sided Pr > |Z|	0.0759

Median One-Way Analysis

Chi-Square	3.1504
DF	1
Pr > Chi-Square	0.0759

NONPARAMETRIC COMPARISON OF BULLFROG LENGTH BETWEEN REFERENCE AND EXPERIMENTAL SITES 1426

-- SEX=M STAGE=A --

The NPAR1WAY Procedure

Wilcoxon Scores (Rank Sums) for Variable SVL
Classified by Variable TYPE

TYPE	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
EXP	156	15553.50	15054.0	300.536458	99.701923
REF	36	2974.50	3474.0	300.536458	82.625000

Average scores were used for ties.

Wilcoxon Two-Sample Test

Statistic	2974.5000
Normal Approximation	
Z	-1.6604
One-Sided Pr < Z	0.0484
Two-Sided Pr > |Z|	0.0968
t Approximation	
One-Sided Pr < Z	0.0492
Two-Sided Pr > |Z|	0.0985

Z includes a continuity correction of 0.5.

Kruskal-Wallis Test

Chi-Square	2.7623
DF	1
Pr > Chi-Square	0.0965

Median Scores (Number of Points Above Median) for Variable SVL
Classified by Variable TYPE

TYPE	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
EXP	156	81.0	78.0	2.711233	0.519231
REF	36	15.0	18.0	2.711233	0.416667

Average scores were used for ties.

Median Two-Sample Test

Statistic	15.0000
Z	-1.1065
One-Sided Pr < Z	0.1343
Two-Sided Pr > |Z|	0.2685

Median One-Way Analysis

Chi-Square	1.2244
DF	1
Pr > Chi-Square	0.2685

NONPARAMETRIC COMPARISON OF BULLFROG LENGTH BETWEEN REFERENCE AND EXPERIMENTAL SITES 1428

-------------------------------------- SEX=UNK STAGE=A --

The NPAR1WAY Procedure

Wilcoxon Scores (Rank Sums) for Variable SVL
Classified by Variable TYPE

TYPE	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
EXP	12	102.0	96.0	6.928203	8.50
REF	3	18.0	24.0	6.928203	6.00

Wilcoxon Two-Sample Test

Statistic	18.0000
Normal Approximation	
Z	-0.7939
One-Sided Pr < Z	0.2136
Two-Sided Pr > |Z|	0.4273
t Approximation	
One-Sided Pr < Z	0.2203
Two-Sided Pr > |Z|	0.4405

Z includes a continuity correction of 0.5.

Kruskal-Wallis Test

Chi-Square	0.7500
DF	1
Pr > Chi-Square	0.3865

Median Scores (Number of Points Above Median) for Variable SVL
Classified by Variable TYPE

TYPE	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
EXP	12	6.0	5.60	0.80	0.500000
REF	3	1.0	1.40	0.80	0.333333

Median Two-Sample Test

Statistic	1.0000
Z	-0.5000
One-Sided Pr < Z	0.3085
Two-Sided Pr > |Z|	0.6171

Median One-Way Analysis

Chi-Square	0.2500
DF	1
Pr > Chi-Square	0.6171

MEAN GONADOSOMATIC INDEX BY STUDY TYPE (REFERENCE VS EXPERIMENTAL), SEX (MALE VS FEMALE), A STAGE (ADULT VS JUVENILE)

Obs	TYPE	SEX	STAGE	_TYPE_	_FREQ_	GSI	STD	CV
1	EXP	F	A	0	15	0.92800	0.74356	80.1245
2	EXP	F	J	0	15	0.60133	0.29384	48.8645
3	EXP	M	A	0	15	0.06467	0.01552	24.0041
4	EXP	M	J	0	10	0.03500	0.01780	50.8432
5	REF	F	A	0	3	1.01000	0.52374	51.8551
6	REF	F	J	0	3	0.66667	0.15822	23.7329
7	REF	M	A	0	6	0.06833	0.01602	23.4451
8	REF	M	J	0	2	0.05000	0.02828	56.5685

NONPARAMETRIC COMPARISON OF GSI BETWEEN REFERENCE AND EXPERIMENTAL SITES 1431

-------------------- SEX=F STAGE=A --------------------

The NPAR1WAY Procedure

Wilcoxon Scores (Rank Sums) for Variable GSI
Classified by Variable TYPE

TYPE	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
EXP	15	138.0	142.50	8.432256	9.20
REF	3	33.0	28.50	8.432256	11.00

Average scores were used for ties.

Wilcoxon Two-Sample Test

Statistic	33.0000
Normal Approximation	
Z	0.4744
One-Sided Pr > Z	0.3176
Two-Sided Pr > |Z|	0.6352
t Approximation	
One-Sided Pr > Z	0.3206
Two-Sided Pr > |Z|	0.6413

Z includes a continuity correction of 0.5.

Kruskal-Wallis Test

Chi-Square	0.2848
DF	1
Pr > Chi-Square	0.5936

Median Scores (Number of Points Above Median) for Variable GSI
Classified by Variable TYPE

TYPE	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
EXP	15	7.0	7.50	0.813489	0.466667
REF	3	2.0	1.50	0.813489	0.666667

Average scores were used for ties.

Median Two-Sample Test

Statistic	2.0000
Z	0.6146
One-Sided Pr > Z	0.2694
Two-Sided Pr > |Z|	0.5388

Median One-Way Analysis

Chi-Square	0.3778
DF	1
Pr > Chi-Square	0.5388

--------------------------------------- SEX=F STAGE=J ---------------------------------------

The NPAR1WAY Procedure

Wilcoxon Scores (Rank Sums) for Variable GSI
Classified by Variable TYPE

TYPE	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
EXP	15	135.50	142.50	8.436615	9.033333
REF	3	35.50	28.50	8.436615	11.833333

Average scores were used for ties.

Wilcoxon Two-Sample Test

Statistic	35.5000
Normal Approximation	
Z	0.7705
One-Sided Pr > Z	0.2205
Two-Sided Pr > |Z|	0.4410
t Approximation	
One-Sided Pr > Z	0.2258
Two-Sided Pr > |Z|	0.4516

Z includes a continuity correction of 0.5.

Kruskal-Wallis Test

Chi-Square	0.6884
DF	1
Pr > Chi-Square	0.4067

Median Scores (Number of Points Above Median) for Variable GSI
Classified by Variable TYPE

TYPE	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
EXP	15	7.0	7.50	0.813489	0.466667
REF	3	2.0	1.50	0.813489	0.666667

Average scores were used for ties.

Median Two-Sample Test

Statistic	2.0000
Z	0.6146
One-Sided Pr > Z	0.2694
Two-Sided Pr > |Z|	0.5388

Median One-Way Analysis

Chi-Square	0.3778
DF	1
Pr > Chi-Square	0.5388

-- SEX=M STAGE=A --

The NPAR1WAY Procedure

Wilcoxon Scores (Rank Sums) for Variable GSI
Classified by Variable TYPE

TYPE	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
EXP	15	155.50	165.0	12.575486	10.366667
REF	6	75.50	66.0	12.575486	12.583333

Average scores were used for ties.

Wilcoxon Two-Sample Test

Statistic	75.5000
Normal Approximation	
Z	0.7157
One-Sided Pr > Z	0.2371
Two-Sided Pr > |Z|	0.4742
t Approximation	
One-Sided Pr > Z	0.2412
Two-Sided Pr > |Z|	0.4825

Z includes a continuity correction of 0.5.

Kruskal-Wallis Test

Chi-Square	0.5707
DF	1
Pr > Chi-Square	0.4500

Median Scores (Number of Points Above Median) for Variable GSI
Classified by Variable TYPE

TYPE	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
EXP	15	5.666667	7.142857	0.971534	0.377778
REF	6	4.333333	2.857143	0.971534	0.722222

Average scores were used for ties.

Median Two-Sample Test

Statistic	4.3333
Z	1.5194
One-Sided Pr > Z	0.0643
Two-Sided Pr > |Z|	0.1287

Median One-Way Analysis

Chi-Square	2.3087
DF	1
Pr > Chi-Square	0.1287

NONPARAMETRIC COMPARISON OF GSI BETWEEN REFERENCE AND EXPERIMENTAL SITES 1437

-- SEX=M STAGE=J --

The NPAR1WAY Procedure

Wilcoxon Scores (Rank Sums) for Variable GSI
Classified by Variable TYPE

TYPE	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
EXP	10	60.50	65.0	4.522670	6.050
REF	2	17.50	13.0	4.522670	8.750

Average scores were used for ties.

Wilcoxon Two-Sample Test

Statistic	17.5000
Normal Approximation	
Z	0.8844
One-Sided Pr > Z	0.1882
Two-Sided Pr > |Z|	0.3765
t Approximation	
One-Sided Pr > Z	0.1977
Two-Sided Pr > |Z|	0.3954

Z includes a continuity correction of 0.5.

Kruskal-Wallis Test

Chi-Square	0.9900
DF	1
Pr > Chi-Square	0.3197

Median Scores (Number of Points Above Median) for Variable GSI
Classified by Variable TYPE

TYPE	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
EXP	10	4.666667	5.0	0.594588	0.466667
REF	2	1.333333	1.0	0.594588	0.666667

Average scores were used for ties.

Median Two-Sample Test

Statistic	1.3333
Z	0.5606
One-Sided Pr > Z	0.2875
Two-Sided Pr > |Z|	0.5751

Median One-Way Analysis

Chi-Square	0.3143
DF	1
Pr > Chi-Square	0.5751

Data Evaluation Report on the Reconnaissance Survey of South Florida Amphibians for the Assessment of Potential Atrazine Effects

EPA MRID Number 458677-06

Data Requirement::

EPA DP Barcode D288775

EPA MRID 458677-06
EPA Guideline 70-1(Special Study)

Test material: **Purity:** {............}
Common name: Atrazine
Chemical name: IUPAC
CAS name 6-chloro-N-ethyl-N'-(1-methylethyl)-1,3,5-triazine-2,4-diamine
CAS No. 1912-24-9
Synonyms
EPA PC Code: 80803

Thomas M Steeger

Primary Reviewer: Thomas M. Steeger, Ph.D., Senior Biologist **Date:** March 24, 2003
Environmental Fate and Effects Division, ERB 4,
U. S. Environmental Protection Agency

Joseph E. Tietge

Secondary Reviewer(s): Joseph E. Tietge, M.S., Research Aquatic Biologist **Date:** April 16, 2003
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Mary Frankenberry

Mary J. Frankenberry, Statistician **Date:** 4/28/03
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Effects in Florida Ecosystems Dominated by Sugarcane Agriculture: A Reconnaissance Survey of Amphibians in South Florida for the Assessment of Potential Atrazine Effects. Department of Physiological Sciences, University of Florida, Caribbean Science Center, Gainesville, Florida. Sponsor: Syngenta Crop Protection, Inc. Study ID: ECORISK Number UFL-02.

EXECUTIVE SUMMARY:

A reconnaissance survey for cane toads (*Bufo marinus*) in south Florida sugarcane-dominated agricultural sites (Belle Glade and Canal Point) and nonagricultural sites (University of Miami) indicated increased incidence of intersex (ovarian tissue in Bidder's organ) in frogs identified as having testes. *B. marinus* is typically a sexually dimorphic amphibian; however, 100% of the cane toads collected at Belle Glade and roughly 55% of the male/intersex frogs collected at Canal Point exhibited female coloration. Additionally, males typically exhibit nuptual pads; however 71% and 0% of the of the intersex frogs collected from Canal Point and Belle Glade, respectively, had nuptual pads. Vitellogenin, a female-specific protein, is not typically expressed in males; however, intersex frogs had vitellogenin levels (774 ± 29 PO_4/mg protein) similar to the females (853 ± 34 PO_4/mg protein) and was roughly double that of male toads (375 ± 34 PO_4/mg protein). Plasma sex steroids (17-β estradiol and testosterone) were relatively gender-specific; however, testosterone levels in intersex males exhibited roughly twice the amount of variability as similar estimates for males. Although agricultural sites had atrazine concentrations ranging from < 0.01 to 24.45 µg/L over the six-month sampling period, no atrazine levels were measured at the University of Miami (nonagricultural) site.

The study authors clearly recognized the potential for other chemicals to confound the interpretation of this study, but they failed to monitor for any other chemicals. The study authors also failed to characterize atrazine levels in nonagricultural sites and water quality parameters that could have impacted the development of frogs.

In this study, the southern toad (*B. terrestris*) was also examined and found to have an increased incidence of intersex (Bidder's organ containing ovarian tissue) in both agricultural and nonagricultural sites. The study authors speculate that the presence of a Bidder's organ may have rendered the animals more sensitive to developmental effects. Bidder's organ is characterized as a nonfunctional, rudimentary ovary; however, no information is available on whether the organ has an endocrine function at any time during the development of the animal. Additionally, while bufonids typically exhibit Bidder's organs at the anterior of each testis, oogenesis is normally abortive because the oocytes fail to reach vitellogenesis and undergo a degenerative process. However, the one slide depicting an intersex male in this study suggested that the oocytes possessed yolk and were therefore vetellogenic.

This study was useful in identifying the incidence of hermaphroditism in field-collected toads. As with the previous studies, toads with testes also appeared to have ovarian tissue, but unlike previous studies the ovarian tissue was associated with the Bidder's organ rather than the testes. While toads collected in agricultural sites may have been exposed to atrazine during development, it is unknown whether atrazine was present at the nonagricultural sites. Other problems with the the study design include exposure to other agrochemicals and environmental conditions relevant to development of toads, *e.g.*, water quality characteristics. These factors limit an interpretation of the findings and make it difficult to establish causality. Based on these data, it is difficult to conclude that atrazine exposure was associated with an increased incidence of intersex. Also, the study does not provide insights on the ecological relevance of the data. If toads depend on coloration to attract mates, though, toads from agricultural sites may have an impaired ability to attract mates because of their distinctly female appearance.

I. MATERIALS AND METHODS

GUIDELINE FOLLOWED: Nonguideline Study

COMPLIANCE: Not conducted under GLP; however, most practices as defined by 40CFR Part 160, August 19, 1989, were established for this study, including but not limited to:
1. Written, authorized preliminary protocol
2. Written, authorized Standard Operating Procedures for all key procedures.
3. Organization and personnel were sufficient in terms of number, education, training and experience.
4. Independent QA inspections were conducted
5. Final Report was written
6. Raw data, documentation, records, protocols, and final report were archived

A. MATERIALS:

1. Test Material [Complete this subsection using the information provided in the methodology section of the study report. Name of test material as cited in the study report.]

Description: Atrazine

Lot No./Batch No. :

Purity: NA

Stability of compound under test conditions:

Storage conditions of test chemicals:_ NA

2. Test organism:

Species: Cane toad (*Bufo marinus*); Southern toad (*B. terrestris*); Green Treefrog (*Hyla cinera)*; Cuban Treefrog (*Osteopilus. septentrionalis*)

Age at test initiation: Field collected animals; age not determined

Weight at study initiation: (mean and range)	Male:	125.1 ± 8.3 g
	Female:	133.9 ± 10.3 g
	Intersex:	123.0 ± 14.5 g
Length at study initiation: (mean and range)	Male:	100.6 ±2.5 mm

Female: 104.9 ± 2.4 mm
Intersex: 99.9 ± 6.7 mm

Source: Field-collected at Belle Glade and Canal Point (sugar cane sites); University of Miami (nonagricultural site)

B. STUDY DESIGN:

Objective:

1) To determine whether exposure of frogs to sugarcane agricultural areas in South Florida would result in a higher incidence of intersex and/or other gonadal/developmental anomalies

2) to examine amphibian populations exposed to a complex mixture of several pesticides and assorted agrichemicals.

1. Experimental Conditions

a) Range-finding Study: Preliminary

b) Definitive Study

Table 1 . Experimental Parameters

Parameter	Details
Acclimation: period: Conditions: (same as test or not) Feeding: Health: (any mortality observed)	NA
Duration of the test	NA
Test condition static/flow- through Type of dilution system for flow- through method. Renewal rate for static renewal	NA
Aeration, if any	NA
Test vessel	NA

Parameter	Details
Source of dilution water Quality:	NA
Water parameters: Hardness pH Dissolved oxygen Total Organic carbon Particulate Matter Metals Pesticides Chlorine Temperature {Salinity for marine or estuarine species} Intervals of water quality measurement	NA
Number of replicates/groups: control: solvent control: treated ones:	NA
Number of organisms per replicate /groups: control: solvent control: treated ones:	NA
Biomass loading rate	NA
Test concentrations: nominal: measured:	NA
Solvent (type, percentage, if used)	NA
Lighting	NA
Feeding	NA
Recovery of chemical Level of Quantitation Level of Detection	NA

Parameter	Details
Positive control {if used, indicate the chemical and concentrations}	NA
Other parameters, if any	NA

Reconnaissance survey was conducted in the field:

Initially, distribution and concentration of atrazine at south Florida sites (Belle Glade and Canal Point) was measured by collecting multiple water samples from several canals/ditches every 2 weeks from February through June with an additional sample in mid-July. A scoping survey of atrazine did not include University of Miami site. No chemicals other than atrazine were screened.

A scoping survey of amphibians for *Bufo marinus* was conducted; however, only the University of Miami site was identified as nonagricultural and having B. marinus.

Although *B. terrestris* was located at Belle Glade, it was not located at Canal Point. Two nonagricultural sites (Archibald Biological Station and Fisheater Creek) did have the Southern Toad.

2. Observations:

Table 2: Observations

Criteria	Details
Parameters measured including the sublethal effects/toxicity symptoms	
Observation intervals	
Were raw data included?	
Other observations, if any	

II. RESULTS and DISCUSSION: [All results discussed in this section and the next are those reported by the study authors. Although supplemental data are typically used in a qualitative manner only, EFED verified spreadsheet data and ran basic statistical analyses on the major study parameters. See attached appendix. If results appeared to differ in any substantive way, the difference was reported in the text below.]

Canals and ditches were sampled for atrazine at both of the agricultural sites. Atrazine levels ranged from < 0.10 to 24.45 ppb at Belle Glade and from < 0.10 to 19.54 ppb at Canal Point. Atrazine concentration at Belle Glade (consisting of 8 sampling sites) peaked in early February (February 10) and then rapidly declined to a more or less steady state for the remainder of the sampling period. At Canal Point (consisting of 8 sampling sites), atrazine concentrations peaked on March 16 and again on April 14, but remained sporadic.

Distribution of Amphibians

Bufo marinus was located in two sugarcane areas (Belle Glade and Canal Point) and only one nonagricultural area, *i.e.*, University of Miami. *Bufo terrestris* was located in one sugarcane area (Belle Glade) and two nonagricultural sites, *i.e.*, Archibald Biological Station and Fisheater Creek. *Hyla cinera* was present at Belle Glade and Archibald. *O. septentrionalis* was present at Belle Glade and Fisheating Creek.

Body weight, length and coloration were recorded; blood was collected by cardiac puncture.

Gender defined by:

female: present of ovarian tissue and absence of testicular tissue
male: presence of testes and absence of developing eggs and/or ovarian tissue
intersex: presence of testes and developing eggs and/or ovarian tissue.

Gonadal Deformities:

segmented testes: testes with clear demarcations or splits
highly segmented: testes with demarcations making testes appear multiple
abnormal shaped: twisted or curled
multiple testes: not defined in text

intersex: not defined in text but likely refers to mix of ovarian and testicular tissue in same organism

Only testes were examined histologically; sections were fixed in formalin and then embedded in parafin. Approximately 29% of the males collected from Belle Glade and 39% of the males collected from Canal Point were intersexed based on ovarian tissue located in the Bidder's organ of males.

For *B. terristris*, 6 of 18 (33%) were intersex from Archibaold (nonagricultural site); 3 of 21 (14%) were intersex from Belle Glade; and 5 of 23 (22%) were intersex from Fisheater Creek (cattle/citrus site).

Plasma was analyzed for 17-β estradiol and testosterone using radioimmunoassay and for alkali-labile phosphate as in indirect measure of vitellogenin.

Table 3 . Distribution of amphibians in both agricultural (sugarcane) and nonagricultural sites.

Species	Agricultural (Sugarcane Site)		Nonagricultural Site		
	Belle Glade	Canal Point	Univ. of Miami	Archibald	Fisheater
Cane Toad (*B. marinus*)	X	X	X		
Southern Toad (*B. terrestris*)	X			X	X
Green Treefrog (*Hyla cinera*)	X			X	
Cuban Treefrog (*Ostropilus septentrionalis*)	X				X

C. VERIFICATION OF STATISTICAL RESULTS: Basic statistics run using SAS® (Statistical Analysis System, Release 8.01, Cary, North Carolina). (See attached printout).

D. STUDY DEFICIENCIES: The study failed to measure atrazine in nonagricultural sites and to collect any information on other chemicals present at the sites. The study also failed to characterize water quality at any of the collection sites.

The University of Miami nonagricultural site was added late in the study and was therefore not included in any of the atrazine sampling efforts.

According to the protocol, animals collected will include: tadpoles at early, mid, and late metamorphosis (>30 per site), metamorphs (>30 per site), and adults (10 males and 10 females per site) as available for each species. Each site will contain 3 replicates/location. Blood will be collected for subsequent endocrine biomarker analysis (E, T, P and DHT). Gonadal tissue from adults will be fixed for subsequent evaluation of gonadal tissues and laryngeal development and subsequent atrazine analysis. The protocol was amended to show that only adults were collected because tadpoles were "difficult to collect." Does this mean that tadpoles were too few in number to collect?

No effort was made to characterize the larynxes because the technique for doing so had not been validated for *B. marinus*.

No P or DHT analysis was performed for *B. marinus* because validated procedures were not available for this species.

Proposed experimental start date on UFL-02 protocol (April 1, 2001) does not agree with proposed experimental start and termination date, i.e., March 1, 2002, and October 1, 2003, respectively also reported in the protocol

E. <u>REVIEWER'S COMMENTS</u>:

Although the study objective states that a range of chemicals are used on sugarcane and implies a recognition that other chemicals would potentially confound any effort to link anomalies to atrazine, the study failed to measure any chemicals in the nonagricultural (University of Miami) site and only measured atrazine in each of the agricultural sites (Belle Glade and Canal Point). Atrazine concentrations in agricultural sites ranged over three orders of magnitude. While some sampling sites exhibited relatively high atrazine concentrations during a sampling date, others samples collected from different areas of the same site exhibited low atrazine concentrations. Although no intersex animals were detected in the nonagricultural site, the authors suggested that the incidence of intersex in agricultural sites may be reflective of "background". The authors also suggested that Bidder's organ may potentially render Bufonidae more susceptible to gonad effects. It is unclear whether the characterization of Bidder's organ as being a "nonfunctional, rudimentary ovary" is entirely correct because literature suggests that if normal males are castrated, the Bidder's organ will differentiate into an ovary. If the theory that atrazine up-regulates is correct and testosterone is transformed into estrogen, is there sufficiently low testosterone or sufficiently high estrogen to result in differentiation of the Bidder's organ?

Across treatment sites, estradiol in intersex animals was only slightly (302 ± 33 pg/mL) above levels found in normal males (293 ± 23 pg/mL); serum testosterone in intersex males was slightly lower (503 ± 307 pg/mL) than normal males (552 ± 174 pg/mL). It is clear, though, that the variability in testosterone levels for intersex males (CV= 61%) was roughly double that of normal males (31.5%). Additionally, labile phosphate as an indirect measure of plasma vitellogenin levels in intersex males (774 ± 29 PO_4/mg protein) was roughly double the level in normal males (375 ± 34 PO_4/mg protein) and was similar to the levels contained in females (853 ± 34 PO_4/mg protein). If the labile phosphate (ALP) is indicative of vitellogenin, then vitellogenesis in intersex animals has been up-regulated by exposure to either an endogenous and/or exogenous estrogen.

It is unclear why the bidder's organ makes the toad a generally more sensitive species to the effects of atrazine or other endocrine disrupting chemicals. It could be argued that this natural hermaphroditic condition is a confounding characteristic since the ovarian tissue of bidder's organ is presumably capable of estrogen synthesis and therefore capable of inducing vitellogenin synthesis in the absence of an exogenous estrogen. This study seems to indicate that vitellogenin levels in the intersex organisms approximates the normal female condition in the absence of elevated plasma estrogen. But, the lack of a strong vitellogenic response of the normal males from the same site suggests that they have not been exposed to an estrogenic chemical. This presumes that the ALP-measurement is a good surrogate for vitellogenin and that the male levels at the reference site represents baseline vitellogenin concentrations. This issue clearly needs more research.

Furthermore, although *Bufo marinus* is typically a sexually dimorphic species with gender-specific coloration being detected in the nonagricultural site collected animals, female coloration pattern was detected in 100% of the frogs (male, female and intersex) collected at Belle Glade and 57%, 54% and 100% of the intersex, male and female frogs collected from Canal Point, respectively.

Nuptual pads were not identified for females from any of the sites. In males collected from nonagricultural sites, 90% had nuptual pads, while 82% and 32% of the males had nuptual pads from Canal Point and Belle Glade, respectively. For males identified as intersex from Canal Point and Belle Glade, 71% and 0% had nuptual pads, respectively.

Testicular abnormalities other than intersex were somewhat similar across collection sites with 44% for Canal Point, 61% for Belle Glade and 45% for University of Miami.

F. CONCLUSIONS: It is not clear why the authors concluded that *Bufo marinus* may be more sensitive to factors that influence gonadal development and sexual differentiation because it possesses a nonfunctional rudimentary ovary (Bidder's organ). For that reason, the species may be a useful sentinel for factors influencing gonadal development. Additionally, while bufonids typically exhibit Bidder's organs at the anterior of each testis, oogenesis is normally abortive because the oocytes fail to reach vitellogenesis and undergo a degenerative process (Petrini and Zaccanti 1998)[1]. However, the one slide (Figure 3F) depicting an intersex male in this study suggests that the oocytes possessed yolk and were therefore vitellogenic. Although the authors state that previous studies have hypothesized a potential developmental mechanism of action for atrazine and that the current study was indirectly designed to evaluate a similar mechanism of action in amphibians, the current study's focus on adults does not permit direct evaluation of this effect. Also, it is difficult to test the hypothesis that atrazine exposure resulted in developmental effects in amphibians because atrazine was not measured in nonagricultural sites, other chemicals were not measured at any of the sites, and no water quality data were collected to characterize the study sites. While it may have been possible to derive some correlation between the incidence of intersex in males and atrazine patterns within particular areas of the sample sites, these data are not provided.

Since intersex, *i.e.*, ovarian tissue in the Bidder's organ,did not appear to involve the testes per se, it is unclear whether the reproductive capacity of the adult frogs was diminished due to this effect. However, the intersex frogs appear to be responding to an environmental estrogen given their elevated vitellogenin levels

[1]Petrini, S. and F. Zaccanti. 1998. The effects of Aromatize and 5 α-reductase inhibitors, antiandrogen, and sex steroids on Bidder's organs development and gonadal differentiation in *Bufo bufo* tadpoles. The Journal of Experimental Zoology 280: 245 - 259.

and a distinctly female appearance. If these frogs rely heavily of coloration to attract mate, their ability to reproduce may be impaired

The incidence of intersex in *Bufo terrestris* also appeared to be elevated; however this was true for both agricultural and nonagricultural sites. The authors speculate that this increased incidence may be indicative of the variability in the background occurrence of intersex among amphibians with a Bidder's organ. Since atrazine levels at the nonagricultural sites were not characterized, it is difficult to speculate on the relevancy of the proposition of "background".

MEAN LENGTH WEIGHT GONAD AND GONADOSOMATIC INDEX BY SITE AND SEX

1

Obs	site	Sex	_TYPE_	_FREQ_	LENGTH	WEIGHT	GONAD	GSE	SD_LT	SD_WT	SD_GONAD	SD_GSI
1	BG	F	0	19	92.979	94.005	2.22374	1.11278	19.0811	67.773	4.3402	1.16438
2	BG	I	0	9	96.332	94.639	0.21622	0.23778	10.6652	40.275	0.0927	0.08913
3	BG	M	0	22	90.763	79.609	0.23955	0.31182	7.4531	21.577	0.2006	0.29333
4	CP	F	0	37	109.067	143.762	6.29178	3.35892	15.8198	65.904	10.8004	5.11631
5	CP	I	0	7	104.639	159.486	0.53771	0.37286	39.9476	59.198	0.1515	0.18901
6	CP	M	0	11	106.042	128.105	0.41200	0.32727	10.1743	47.890	0.1693	0.11934
7	UM	F	0	4	122.835	232.685	9.56100	4.33500	13.9482	136.862	5.1713	1.41630
8	UM	M	0	20	108.400	174.345	0.40530	0.23050	24.0066	57.599	0.1495	0.05375

ANOVA FOR LENGTH BETWEEN SITES 2

--- Sex=F ---

The ANOVA Procedure

Class Level Information

Class	Levels	Values
site	3	BG CP UM

Number of observations 60

Dependent Variable: SVL

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	2	4629.32548	2314.66274	8.17	0.0008
Error	57	16146.86161	283.27827		
Corrected Total	59	20776.18709			

R-Square	Coeff Var	Root MSE	SVL Mean
0.222819	16.04614	16.83087	104.8905

Source	DF	Anova SS	Mean Square	F Value	Pr > F
site	2	4629.325476	2314.662738	8.17	0.0008

Levene's Test for Homogeneity of SVL Variance
ANOVA of Squared Deviations from Group Means

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
site	2	194186	97093.2	0.56	0.5751
Error	57	9906646	173801		

Bartlett's Test for Homogeneity of SVL Variance

Source	DF	Chi-Square	Pr > ChiSq
site	2	1.0035	0.6055

Dunnett's t Tests for SVL

NOTE: This test controls the Type I experimentwise error for comparisons of all treatments against a control.

Alpha	0.05
Error Degrees of Freedom	57
Error Mean Square	283.2783
Critical Value of Dunnett's t	2.28365

Comparisons significant at the 0.05 level are indicated by ***.

site Comparison	Difference Between Means	Simultaneous 95% Confidence Limits		
UM - BG	29.856	8.712	51.000	***
CP - BG	16.088	5.240	26.936	***

-- Sex=I --

The ANOVA Procedure

Class Level Information

Class	Levels	Values
site	2	BG CP

Number of observations 16

Dependent Variable: SVL

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	1	271.66953	271.66953	0.36	0.5566
Error	14	10484.85064	748.91790		
Corrected Total	15	10756.52018			

R-Square	Coeff Var	Root MSE	SVL Mean
0.025256	27.37560	27.36636	99.96625

Source	DF	Anova SS	Mean Square	F Value	Pr > F
site	1	271.6695337	271.6695337	0.36	0.5566

Levene's Test for Homogeneity of SVL Variance
ANOVA of Squared Deviations from Group Means

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
site	1	6318154	6318154	1.83	0.1976
Error	14	48349862	3453562		

Bartlett's Test for Homogeneity of SVL Variance

Source	DF	Chi-Square	Pr > ChiSq
site	1	9.8175	0.0017

Dunnett's t Tests for SVL

NOTE: This test controls the Type I experimentwise error for comparisons of all treatments against a control.

Alpha	0.05
Error Degrees of Freedom	14
Error Mean Square	748.9179
Critical Value of Dunnett's t	2.14485

Comparisons significant at the 0.05 level are indicated by ***.

site Comparison	Difference Between Means	Simultaneous 95% Confidence Limits	
CP - BG	8.306	-21.274	37.887

ANOVA FOR LENGTH BETWEEN SITES 10

-- Sex=M --

The ANOVA Procedure

Class Level Information

Class	Levels	Values
site	3	BG CP UM

Number of observations 53

Dependent Variable: SVL

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	2	3671.52388	1835.76194	6.98	0.0021
Error	50	13151.65680	263.03314		
Corrected Total	52	16823.18068			

R-Square	Coeff Var	Root MSE	SVL Mean
0.218242	16.12326	16.21830	100.5894

Source	DF	Anova SS	Mean Square	F Value	Pr > F
site	2	3671.523883	1835.761942	6.98	0.0021

ANOVA FOR LENGTH BETWEEN SITES 12

Levene's Test for Homogeneity of SVL Variance
ANOVA of Squared Deviations from Group Means

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
site	2	2890854	1445427	0.89	0.4168
Error	50	81136179	1622724		

Bartlett's Test for Homogeneity of SVL Variance

Source	DF	Chi-Square	Pr > ChiSq
site	2	26.2881	<.0001

Dunnett's t Tests for SVL

NOTE: This test controls the Type I experimentwise error for comparisons of all treatments against a control.

Alpha	0.05
Error Degrees of Freedom	50
Error Mean Square	263.0331
Critical Value of Dunnett's t	2.28707

Comparisons significant at the 0.05 level are indicated by ***.

site Comparison	Difference Between Means	Simultaneous 95% Confidence Limits		
UM - BG	17.637	6.177	29.097	***
CP - BG	15.279	1.582	28.976	***

NONPARAMETRIC COMPARISON OF LENGTH BETWEEN SITES 14

-- Sex=F --

The NPAR1WAY Procedure

Wilcoxon Scores (Rank Sums) for Variable SVL
Classified by Variable site

site	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
BG	19	363.0	579.50	62.927869	19.105263
CP	37	1276.0	1128.50	65.771701	34.486486
UM	4	191.0	122.00	33.744135	47.750000

Kruskal-Wallis Test

Chi-Square	13.9187
DF	2
Pr > Chi-Square	0.0009

Median Scores (Number of Points Above Median) for Variable SVL
Classified by Variable site

site	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
BG	19	4.0	9.50	1.816823	0.210526
CP	37	22.0	18.50	1.898929	0.594595
UM	4	4.0	2.00	0.974245	1.000000

Median One-Way Analysis

Chi-Square	11.4979
DF	2
Pr > Chi-Square	0.0032

NONPARAMETRIC COMPARISON OF LENGTH BETWEEN SITES 16

--- Sex=I ---

The NPAR1WAY Procedure

Wilcoxon Scores (Rank Sums) for Variable SVL
Classified by Variable site

site	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
BG	9	56.0	76.50	9.447222	6.222222
CP	7	80.0	59.50	9.447222	11.428571

Wilcoxon Two-Sample Test

Statistic	80.0000
Normal Approximation	
Z	2.1170
One-Sided Pr > Z	0.0171
Two-Sided Pr > |Z|	0.0343
t Approximation	
One-Sided Pr > Z	0.0257
Two-Sided Pr > |Z|	0.0514

Z includes a continuity correction of 0.5.

Kruskal-Wallis Test

Chi-Square	4.7087
DF	1
Pr > Chi-Square	0.0300

The NPAR1WAY Procedure

Median Scores (Number of Points Above Median) for Variable SVL
Classified by Variable site

site	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
BG	9	2.0	4.50	1.024695	0.222222
CP	7	6.0	3.50	1.024695	0.857143

Median Two-Sample Test

Statistic	6.0000
Z	2.4398
One-Sided Pr > Z	0.0073
Two-Sided Pr > |Z|	0.0147

Median One-Way Analysis

Chi-Square	5.9524
DF	1
Pr > Chi-Square	0.0147

NONPARAMETRIC COMPARISON OF LENGTH BETWEEN SITES 18

--- Sex=M ---

The NPAR1WAY Procedure

Wilcoxon Scores (Rank Sums) for Variable SVL
Classified by Variable site

site	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
BG	22	313.0	594.0	55.398556	14.227273
CP	11	348.0	297.0	45.596052	31.636364
UM	20	770.0	540.0	54.497706	38.500000

Kruskal-Wallis Test

Chi-Square	27.1304
DF	2
Pr > Chi-Square	<.0001

The NPAR1WAY Procedure

Median Scores (Number of Points Above Median) for Variable SVL
Classified by Variable site

site	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
BG	22	1.0	10.792453	1.810436	0.045455
CP	11	7.0	5.396226	1.490088	0.636364
UM	20	18.0	9.811321	1.780996	0.900000

Median One-Way Analysis

Chi-Square	31.1926
DF	2
Pr > Chi-Square	<.0001

ANOVA FOR WEIGHT BETWEEN SITES 20

--- Sex=F ---

The ANOVA Procedure

Class Level Information

Class	Levels	Values
site	3	BG CP UM

Number of observations 60

Dependent Variable: wt

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	2	72873.3117	36436.6559	7.03	0.0019
Error	57	295228.9890	5179.4559		
Corrected Total	59	368102.3007			

R-Square	Coeff Var	Root MSE	wt Mean
0.197970	53.73440	71.96844	133.9336

Source	DF	Anova SS	Mean Square	F Value	Pr > F
site	2	72873.31174	36436.65587	7.03	0.0019

Levene's Test for Homogeneity of wt Variance
ANOVA of Squared Deviations from Group Means

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
site	2	3.5729E8	1.7864E8	2.91	0.0624
Error	57	3.4941E9	61300449		

Bartlett's Test for Homogeneity of wt Variance

Source	DF	Chi-Square	Pr > ChiSq
site	2	4.3547	0.1133

Dunnett's t Tests for wt

NOTE: This test controls the Type I experimentwise error for comparisons of all treatments against a control.

Alpha	0.05
Error Degrees of Freedom	57
Error Mean Square	5179.456
Critical Value of Dunnett's t	2.28365

Comparisons significant at the 0.05 level are indicated by ***.

site Comparison	Difference Between Means	Simultaneous 95% Confidence Limits		
UM - BG	138.68	48.27	229.09	***
CP - BG	49.76	3.37	96.14	***

-- Sex=I --

The ANOVA Procedure

Class Level Information

Class	Levels	Values
site	2	BG CP

Number of observations 16

Dependent Variable: wt

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	1	16557.62363	16557.62363	6.82	0.0205
Error	14	34003.16446	2428.79746		
Corrected Total	15	50560.78809			

R-Square	Coeff Var	Root MSE	wt Mean
0.327480	40.06429	49.28283	123.0094

Source	DF	Anova SS	Mean Square	F Value	Pr > F
site	1	16557.62363	16557.62363	6.82	0.0205

Levene's Test for Homogeneity of wt Variance
ANOVA of Squared Deviations from Group Means

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
site	1	9605650	9605650	1.11	0.3100
Error	14	1.2117E8	8654926		

Bartlett's Test for Homogeneity of wt Variance

Source	DF	Chi-Square	Pr > ChiSq
site	1	0.9592	0.3274

Dunnett's t Tests for wt

NOTE: This test controls the Type I experimentwise error for comparisons of all treatments against a control.

Alpha	0.05
Error Degrees of Freedom	14
Error Mean Square	2428.797
Critical Value of Dunnett's t	2.14485

Comparisons significant at the 0.05 level are indicated by ***.

site Comparison	Difference Between Means	Simultaneous 95% Confidence Limits		
CP - BG	64.85	11.58	118.12	***

ANOVA FOR WEIGHT BETWEEN SITES 28

--- Sex=M ---

The ANOVA Procedure

Class Level Information

Class	Levels	Values
site	3	BG CP UM

Number of observations 53

Dependent Variable: wt

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	2	94122.4446	47061.2223	24.58	<.0001
Error	50	95747.7804	1914.9556		
Corrected Total	52	189870.2250			

R-Square	Coeff Var	Root MSE	wt Mean
0.495720	34.88999	43.76021	125.4234

Source	DF	Anova SS	Mean Square	F Value	Pr > F
site	2	94122.44456	47061.22228	24.58	<.0001

Levene's Test for Homogeneity of wt Variance
ANOVA of Squared Deviations from Group Means

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
site	2	77865494	38932747	3.38	0.0420
Error	50	5.7584E8	11516787		

Bartlett's Test for Homogeneity of wt Variance

Source	DF	Chi-Square	Pr > ChiSq
site	2	16.9429	0.0002

Dunnett's t Tests for wt

NOTE: This test controls the Type I experimentwise error for comparisons of all treatments against a control.

Alpha	0.05
Error Degrees of Freedom	50
Error Mean Square	1914.956
Critical Value of Dunnett's t	2.28707

Comparisons significant at the 0.05 level are indicated by ***.

site Comparison	Difference Between Means	Simultaneous 95% Confidence Limits		
UM - BG	94.74	63.81	125.66	***
CP - BG	48.50	11.54	85.45	***

-- Sex=F --

The NPAR1WAY Procedure

Wilcoxon Scores (Rank Sums) for Variable wt
Classified by Variable site

site	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
BG	19	371.0	579.50	62.926995	19.526316
CP	37	1270.0	1128.50	65.770787	34.324324
UM	4	189.0	122.00	33.743666	47.250000

Average scores were used for ties.

Kruskal-Wallis Test

Chi-Square	12.9558
DF	2
Pr > Chi-Square	0.0015

Median Scores (Number of Points Above Median) for Variable wt
Classified by Variable site

site	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
BG	19	3.0	9.50	1.816823	0.157895
CP	37	23.0	18.50	1.898929	0.621622
UM	4	4.0	2.00	0.974245	1.000000

Average scores were used for ties.

Median One-Way Analysis

Chi-Square	14.8325
DF	2
Pr > Chi-Square	0.0006

-- Sex=I --

The NPAR1WAY Procedure

Wilcoxon Scores (Rank Sums) for Variable wt
Classified by Variable site

site	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
BG	9	59.0	76.50	9.447222	6.555556
CP	7	77.0	59.50	9.447222	11.000000

Wilcoxon Two-Sample Test

Statistic	77.0000
Normal Approximation	
Z	1.7995
One-Sided Pr > Z	0.0360
Two-Sided Pr > |Z|	0.0719
t Approximation	
One-Sided Pr > Z	0.0460
Two-Sided Pr > |Z|	0.0921

Z includes a continuity correction of 0.5.

Kruskal-Wallis Test

Chi-Square	3.4314
DF	1
Pr > Chi-Square	0.0640

Median Scores (Number of Points Above Median) for Variable wt
Classified by Variable site

site	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
BG	9	3.0	4.50	1.024695	0.333333
CP	7	5.0	3.50	1.024695	0.714286

Median Two-Sample Test

Statistic	5.0000
Z	1.4639
One-Sided Pr > Z	0.0716
Two-Sided Pr > |Z|	0.1432

Median One-Way Analysis

Chi-Square	2.1429
DF	1
Pr > Chi-Square	0.1432

-- Sex=M --

The NPAR1WAY Procedure

Wilcoxon Scores (Rank Sums) for Variable wt
Classified by Variable site

site	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
BG	22	331.0	594.0	55.397439	15.045455
CP	11	313.0	297.0	45.595133	28.454545
UM	20	787.0	540.0	54.496608	39.350000

Average scores were used for ties.

Kruskal-Wallis Test

Chi-Square	26.0714
DF	2
Pr > Chi-Square	<.0001

Median Scores (Number of Points Above Median) for Variable wt
Classified by Variable site

site	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
BG	22	1.0	10.792453	1.810436	0.045455
CP	11	7.0	5.396226	1.490088	0.636364
UM	20	18.0	9.811321	1.780996	0.900000

Average scores were used for ties.

Median One-Way Analysis

Chi-Square	31.1926
DF	2
Pr > Chi-Square	<.0001

--- Sex=F ---

The ANOVA Procedure

Class Level Information

Class	Levels	Values
site	3	BG CP UM

Number of observations 60

Dependent Variable: gonad

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	2	288.453909	144.226955	1.78	0.1779
Error	57	4618.623706	81.028486		
Corrected Total	59	4907.077615			

R-Square	Coeff Var	Root MSE	gonad Mean
0.058783	172.3940	9.001582	5.221517

Source	DF	Anova SS	Mean Square	F Value	Pr > F
site	2	288.4539090	144.2269545	1.78	0.1779

Levene's Test for Homogeneity of gonad Variance
ANOVA of Squared Deviations from Group Means

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
site	2	128737	64368.3	1.67	0.1977
Error	57	2199727	38591.7		

Bartlett's Test for Homogeneity of gonad Variance

Source	DF	Chi-Square	Pr > ChiSq
site	2	15.4430	0.0004

Dunnett's t Tests for gonad

NOTE: This test controls the Type I experimentwise error for comparisons of all treatments against a control.

Alpha	0.05
Error Degrees of Freedom	57
Error Mean Square	81.02849
Critical Value of Dunnett's t	2.28365

Comparisons significant at the 0.05 level are indicated by ***.

site Comparison	Difference Between Means	Simultaneous 95% Confidence Limits	
UM - BG	7.337	-3.971	18.646
CP - BG	4.068	-1.734	9.870

ANOVA FOR GONAD WEIGHT BETWEEN SITES

--- Sex=I ---

The ANOVA Procedure

Class Level Information

Class	Levels	Values
site	2	BG CP

Number of observations 16

Dependent Variable: gonad

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	1	0.40696877	0.40696877	27.59	0.0001
Error	14	0.20647098	0.01474793		
Corrected Total	15	0.61343975			

R-Square	Coeff Var	Root MSE	gonad Mean
0.663421	34.02901	0.121441	0.356875

Source	DF	Anova SS	Mean Square	F Value	Pr > F
site	1	0.40696877	0.40696877	27.59	0.0001

Levene's Test for Homogeneity of gonad Variance
ANOVA of Squared Deviations from Group Means

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
site	1	0.000571	0.000571	0.92	0.3534
Error	14	0.00868	0.000620		

Bartlett's Test for Homogeneity of gonad Variance

Source	DF	Chi-Square	Pr > ChiSq
site	1	1.5542	0.2125

Dunnett's t Tests for gonad

NOTE: This test controls the Type I experimentwise error for comparisons of all treatments against a control.

Alpha	0.05
Error Degrees of Freedom	14
Error Mean Square	0.014748
Critical Value of Dunnett's t	2.14485

Comparisons significant at the 0.05 level are indicated by ***.

site Comparison	Difference Between Means	Simultaneous 95% Confidence Limits		
CP - BG	0.32149	0.19023	0.45276	***

ANOVA FOR GONAD WEIGHT BETWEEN SITES 46

--- Sex=M ---

The ANOVA Procedure

Class Level Information

Class	Levels	Values
site	3	BG CP UM

Number of observations 53

Dependent Variable: gonad

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	2	0.36407367	0.18203683	5.85	0.0052
Error	50	1.55651365	0.03113027		
Corrected Total	52	1.92058732			

R-Square	Coeff Var	Root MSE	gonad Mean
0.189564	52.21800	0.176438	0.337887

Source	DF	Anova SS	Mean Square	F Value	Pr > F
site	2	0.36407367	0.18203683	5.85	0.0052

Levene's Test for Homogeneity of gonad Variance
ANOVA of Squared Deviations from Group Means

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
site	2	0.00324	0.00162	0.27	0.7670
Error	50	0.3041	0.00608		

Bartlett's Test for Homogeneity of gonad Variance

Source	DF	Chi-Square	Pr > ChiSq
site	2	1.6748	0.4328

Dunnett's t Tests for gonad

NOTE: This test controls the Type I experimentwise error for comparisons of all treatments against a control.

Alpha	0.05
Error Degrees of Freedom	50
Error Mean Square	0.03113
Critical Value of Dunnett's t	2.28707

Comparisons significant at the 0.05 level are indicated by ***.

site Comparison	Difference Between Means	Simultaneous 95% Confidence Limits		
CP - BG	0.17245	0.02344	0.32147	***
UM - BG	0.16575	0.04108	0.29043	***

-- Sex=F --

The NPAR1WAY Procedure

Wilcoxon Scores (Rank Sums) for Variable gonad
Classified by Variable site

site	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
BG	19	346.0	579.50	62.926995	18.210526
CP	37	1282.0	1128.50	65.770787	34.648649
UM	4	202.0	122.00	33.743666	50.500000

Average scores were used for ties.

Kruskal-Wallis Test

Chi-Square	16.7428
DF	2
Pr > Chi-Square	0.0002

Median Scores (Number of Points Above Median) for Variable gonad
Classified by Variable site

site	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
BG	19	4.0	9.50	1.816823	0.210526
CP	37	22.0	18.50	1.898929	0.594595
UM	4	4.0	2.00	0.974245	1.000000

Average scores were used for ties.

Median One-Way Analysis

Chi-Square	11.4979
DF	2
Pr > Chi-Square	0.0032

-- Sex=I --

The NPAR1WAY Procedure

Wilcoxon Scores (Rank Sums) for Variable gonad
Classified by Variable site

site	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
BG	9	48.0	76.50	9.433319	5.333333
CP	7	88.0	59.50	9.433319	12.571429

Average scores were used for ties.

Wilcoxon Two-Sample Test

Statistic	88.0000
Normal Approximation	
Z	2.9682
One-Sided Pr > Z	0.0015
Two-Sided Pr > |Z|	0.0030
t Approximation	
One-Sided Pr > Z	0.0048
Two-Sided Pr > |Z|	0.0096

Z includes a continuity correction of 0.5.

Kruskal-Wallis Test

Chi-Square	9.1277
DF	1
Pr > Chi-Square	0.0025

Median Scores (Number of Points Above Median) for Variable gonad
Classified by Variable site

site	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
BG	9	2.0	4.50	1.024695	0.222222
CP	7	6.0	3.50	1.024695	0.857143

Average scores were used for ties.

Median Two-Sample Test

Statistic	6.0000
Z	2.4398
One-Sided Pr > Z	0.0073
Two-Sided Pr > |Z|	0.0147

Median One-Way Analysis

Chi-Square	5.9524
DF	1
Pr > Chi-Square	0.0147

NONPARAMETRIC COMPARISON OF GONAD WEIGHT BETWEEN SITES 54

--- Sex=M ---

The NPAR1WAY Procedure

Wilcoxon Scores (Rank Sums) for Variable gonad
Classified by Variable site

site	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
BG	22	382.50	594.0	55.386271	17.386364
CP	11	377.50	297.0	45.585941	34.318182
UM	20	671.00	540.0	54.485621	33.550000

Average scores were used for ties.

Kruskal-Wallis Test

Chi-Square	14.5995
DF	2
Pr > Chi-Square	0.0007

Median Scores (Number of Points Above Median) for Variable gonad
Classified by Variable site

site	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
BG	22	4.0	10.792453	1.810436	0.181818
CP	11	8.0	5.396226	1.490088	0.727273
UM	20	14.0	9.811321	1.780996	0.700000

Average scores were used for ties.

Median One-Way Analysis

Chi-Square	14.0970
DF	2
Pr > Chi-Square	0.0009

ANOVA FOR GONADOSOMATIC INDEX BETWEEN SITES 56

--- Sex=F ---

The ANOVA Procedure

Class Level Information

Class	Levels	Values
site	3	BG CP UM

Number of observations 60

NOTE: Due to missing values, only 59 observations can be used in this analysis.

Dependent Variable: GSI

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	2	72.010680	36.005340	2.08	0.1350
Error	56	971.423418	17.346847		
Corrected Total	58	1043.434098			

R-Square	Coeff Var	Root MSE	GSI Mean
0.069013	152.0150	4.164955	2.739831

Source	DF	Anova SS	Mean Square	F Value	Pr > F
site	2	72.01068044	36.00534022	2.08	0.1350

Levene's Test for Homogeneity of GSI Variance
ANOVA of Squared Deviations from Group Means

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
site	2	8045.3	4022.6	1.28	0.2865
Error	56	176236	3147.1		

Bartlett's Test for Homogeneity of GSI Variance

Source	DF	Chi-Square	Pr > ChiSq
site	2	32.7955	<.0001

Dunnett's t Tests for GSI

NOTE: This test controls the Type I experimentwise error for comparisons of all treatments against a control.

Alpha	0.05
Error Degrees of Freedom	56
Error Mean Square	17.34685
Critical Value of Dunnett's t	2.28391

Comparisons significant at the 0.05 level are indicated by ***.

site Comparison	Difference Between Means	Simultaneous 95% Confidence Limits	
UM - BG	3.2222	-2.0359	8.4804
CP - BG	2.2461	-0.4875	4.9797

ANOVA FOR GONADOSOMATIC INDEX BETWEEN SITES 60

--- Sex=I ---

The ANOVA Procedure

Class Level Information

Class	Levels	Values
site	2	BG CP

Number of observations 16

Dependent Variable: GSI

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	1	0.07184534	0.07184534	3.62	0.0779
Error	14	0.27789841	0.01984989		
Corrected Total	15	0.34974375			

R-Square	Coeff Var	Root MSE	GSI Mean
0.205423	47.45756	0.140890	0.296875

Source	DF	Anova SS	Mean Square	F Value	Pr > F
site	1	0.07184534	0.07184534	3.62	0.0779

Levene's Test for Homogeneity of GSI Variance
ANOVA of Squared Deviations from Group Means

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
site	1	0.00219	0.00219	1.21	0.2896
Error	14	0.0253	0.00180		

Bartlett's Test for Homogeneity of GSI Variance

Source	DF	Chi-Square	Pr > ChiSq
site	1	3.5402	0.0599

Dunnett's t Tests for GSI

NOTE: This test controls the Type I experimentwise error for comparisons of all treatments against a control.

Alpha	0.05
Error Degrees of Freedom	14
Error Mean Square	0.01985
Critical Value of Dunnett's t	2.14485

Comparisons significant at the 0.05 level are indicated by ***.

site Comparison	Difference Between Means	Simultaneous 95% Confidence Limits	
CP - BG	0.13508	-0.01721	0.28737

ANOVA FOR GONADOSOMATIC INDEX BETWEEN SITES 64

--- Sex=M ---

The ANOVA Procedure

Class Level Information

Class	Levels	Values
site	3	BG CP UM

Number of observations 53

Dependent Variable: GSI

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	2	0.09486143	0.04743072	1.18	0.3147
Error	50	2.00424045	0.04008481		
Corrected Total	52	2.09910189			

R-Square	Coeff Var	Root MSE	GSI Mean
0.045191	70.41295	0.200212	0.284340

Source	DF	Anova SS	Mean Square	F Value	Pr > F
site	2	0.09486143	0.04743072	1.18	0.3147

Levene's Test for Homogeneity of GSI Variance
ANOVA of Squared Deviations from Group Means

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
site	2	0.0746	0.0373	1.60	0.2121
Error	50	1.1661	0.0233		

Bartlett's Test for Homogeneity of GSI Variance

Source	DF	Chi-Square	Pr > ChiSq
site	2	42.9859	<.0001

Dunnett's t Tests for GSI

NOTE: This test controls the Type I experimentwise error for comparisons of all treatments against a control.

Alpha	0.05
Error Degrees of Freedom	50
Error Mean Square	0.040085
Critical Value of Dunnett's t	2.28707

Comparisons significant at the 0.05 level are indicated by ***.

site Comparison	Difference Between Means	Simultaneous 95% Confidence Limits	
CP - BG	0.01545	-0.15364	0.18454
UM - BG	-0.08132	-0.22279	0.06015

NONPARAMETRIC COMPARISON OF GONADOSOMATIC INDEX (GSI) BETWEEN SITES 68

-- Sex=F --

The NPAR1WAY Procedure

Wilcoxon Scores (Rank Sums) for Variable GSI
Classified by Variable site

site	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
BG	18	306.50	540.0	60.741820	17.027778
CP	37	1265.50	1110.0	63.792823	34.202703
UM	4	198.00	120.0	33.164309	49.500000

Average scores were used for ties.

Kruskal-Wallis Test

Chi-Square	17.6412
DF	2
Pr > Chi-Square	0.0001

Median Scores (Number of Points Above Median) for Variable GSI
Classified by Variable site

site	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
BG	18	4.0	8.847458	1.783289	0.222222
CP	37	21.0	18.186441	1.872862	0.567568
UM	4	4.0	1.966102	0.973655	1.000000

Average scores were used for ties.

Median One-Way Analysis

Chi-Square	10.0440
DF	2
Pr > Chi-Square	0.0066

NONPARAMETRIC COMPARISON OF GONADOSOMATIC INDEX (GSI) BETWEEN SITES 70

--- Sex=I ---

The NPAR1WAY Procedure

Wilcoxon Scores (Rank Sums) for Variable GSI
Classified by Variable site

site	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
BG	9	56.50	76.50	9.440273	6.277778
CP	7	79.50	59.50	9.440273	11.357143

Average scores were used for ties.

Wilcoxon Two-Sample Test

Statistic	79.5000
Normal Approximation	
Z	2.0656
One-Sided Pr > Z	0.0194
Two-Sided Pr > |Z|	0.0389
t Approximation	
One-Sided Pr > Z	0.0283
Two-Sided Pr > |Z|	0.0566

Z includes a continuity correction of 0.5.

Kruskal-Wallis Test

Chi-Square	4.4884
DF	1
Pr > Chi-Square	0.0341

Median Scores (Number of Points Above Median) for Variable GSI
Classified by Variable site

site	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
BG	9	2.0	4.50	1.024695	0.222222
CP	7	6.0	3.50	1.024695	0.857143

Average scores were used for ties.

Median Two-Sample Test

Statistic	6.0000
Z	2.4398
One-Sided Pr > Z	0.0073
Two-Sided Pr > |Z|	0.0147

Median One-Way Analysis

Chi-Square	5.9524
DF	1
Pr > Chi-Square	0.0147

NONPARAMETRIC COMPARISON OF GONADOSOMATIC INDEX (GSI) BETWEEN SITES 72

--- Sex=M ---

The NPAR1WAY Procedure

Wilcoxon Scores (Rank Sums) for Variable GSI
Classified by Variable site

site	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
BG	22	524.50	594.0	55.289008	23.840909
CP	11	423.00	297.0	45.505889	38.454545
UM	20	483.50	540.0	54.389940	24.175000

Average scores were used for ties.

Kruskal-Wallis Test

Chi-Square	7.6716
DF	2
Pr > Chi-Square	0.0216

Median Scores (Number of Points Above Median) for Variable GSI
Classified by Variable site

site	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
BG	22	10.00	10.792453	1.758432	0.454545
CP	11	8.50	5.396226	1.447286	0.772727
UM	20	7.50	9.811321	1.729838	0.375000

Average scores were used for ties.

Median One-Way Analysis

Chi-Square	4.8749
DF	2
Pr > Chi-Square	0.0874

-- Sex=F --

The ANOVA Procedure

Class Level Information

Class	Levels	Values
site	3	BG CP UM

Number of observations 60

Dependent Variable: CI

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	2	2.87929138	1.43964569	6.71	0.0024
Error	57	12.22812021	0.21452842		
Corrected Total	59	15.10741159			

R-Square	Coeff Var	Root MSE	CI Mean
0.190588	38.52713	0.463172	1.202197

Source	DF	Anova SS	Mean Square	F Value	Pr > F
site	2	2.87929138	1.43964569	6.71	0.0024

Levene's Test for Homogeneity of CI Variance
ANOVA of Squared Deviations from Group Means

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
site	2	0.6135	0.3067	3.66	0.0320
Error	57	4.7813	0.0839		

Bartlett's Test for Homogeneity of CI Variance

Source	DF	Chi-Square	Pr > ChiSq
site	2	4.3397	0.1142

Dunnett's t Tests for CI

NOTE: This test controls the Type I experimentwise error for comparisons of all treatments against a control.

Alpha	0.05
Error Degrees of Freedom	57
Error Mean Square	0.214528
Critical Value of Dunnett's t	2.28365

Comparisons significant at the 0.05 level are indicated by ***.

site Comparison	Difference Between Means	Simultaneous 95% Confidence Limits		
UM - BG	0.8712	0.2893	1.4531	***
CP - BG	0.3133	0.0148	0.6119	***

ANOVA FOR CONDITION INDEX BETWEEN SITES 78

--- Sex=I ---

The ANOVA Procedure

Class Level Information

Class	Levels	Values
site	2	BG CP

Number of observations 16

Dependent Variable: CI

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	1	8.11930253	8.11930253	2.32	0.1499
Error	14	48.97043484	3.49788820		
Corrected Total	15	57.08973737			

R-Square	Coeff Var	Root MSE	CI Mean
0.142220	118.0374	1.870264	1.584467

Source	DF	Anova SS	Mean Square	F Value	Pr > F
site	1	8.11930253	8.11930253	2.32	0.1499

Levene's Test for Homogeneity of CI Variance
ANOVA of Squared Deviations from Group Means

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
site	1	183.9	183.9	1.95	0.1844
Error	14	1320.7	94.3380		

Bartlett's Test for Homogeneity of CI Variance

Source	DF	Chi-Square	Pr > ChiSq
site	1	23.5316	<.0001

Dunnett's t Tests for CI

NOTE: This test controls the Type I experimentwise error for comparisons of all treatments against a control.

Alpha	0.05
Error Degrees of Freedom	14
Error Mean Square	3.497888
Critical Value of Dunnett's t	2.14485

Comparisons significant at the 0.05 level are indicated by ***.

site Comparison	Difference Between Means	Simultaneous 95% Confidence Limits	
CP - BG	1.4360	-0.5856	3.4576

-- Sex=M --

The ANOVA Procedure

Class Level Information

Class	Levels	Values
site	3	BG CP UM

Number of observations 53

Dependent Variable: CI

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	2	22.6025084	11.3012542	2.26	0.1149
Error	50	250.0105867	5.0002117		
Corrected Total	52	272.6130951			

R-Square	Coeff Var	Root MSE	CI Mean
0.082911	151.8343	2.236115	1.472734

Source	DF	Anova SS	Mean Square	F Value	Pr > F
site	2	22.60250839	11.30125420	2.26	0.1149

Levene's Test for Homogeneity of CI Variance
ANOVA of Squared Deviations from Group Means

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
site	2	1898.9	949.5	0.93	0.4022
Error	50	51177.9	1023.6		

Bartlett's Test for Homogeneity of CI Variance

Source	DF	Chi-Square	Pr > ChiSq
site	2	122.7	<.0001

Dunnett's t Tests for CI

NOTE: This test controls the Type I experimentwise error for comparisons of all treatments against a control.

Alpha	0.05
Error Degrees of Freedom	50
Error Mean Square	5.000212
Critical Value of Dunnett's t	2.28707

Comparisons significant at the 0.05 level are indicated by ***.

site Comparison	Difference Between Means	Simultaneous 95% Confidence Limits	
UM - BG	1.4305	-0.1496	3.0105
CP - BG	0.3156	-1.5730	2.2041

NONPARAMETRIC COMPARISON OF CONDITION INDEX (CI) BETWEEN SITES 86

-- Sex=F --

The NPAR1WAY Procedure

Wilcoxon Scores (Rank Sums) for Variable CI
Classified by Variable site

site	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
BG	19	393.0	579.50	62.927869	20.684211
CP	37	1253.0	1128.50	65.771701	33.864865
UM	4	184.0	122.00	33.744135	46.000000

Kruskal-Wallis Test

Chi-Square	10.5265
DF	2
Pr > Chi-Square	0.0052

Median Scores (Number of Points Above Median) for Variable CI
Classified by Variable site

site	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
BG	19	4.0	9.50	1.816823	0.210526
CP	37	23.0	18.50	1.898929	0.621622
UM	4	3.0	2.00	0.974245	0.750000

Median One-Way Analysis

Chi-Square	9.3983
DF	2
Pr > Chi-Square	0.0091

--- Sex=I ---

The NPAR1WAY Procedure

Wilcoxon Scores (Rank Sums) for Variable CI
Classified by Variable site

site	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
BG	9	60.0	76.50	9.447222	6.666667
CP	7	76.0	59.50	9.447222	10.857143

Wilcoxon Two-Sample Test

Statistic	76.0000
Normal Approximation	
Z	1.6936
One-Sided Pr > Z	0.0452
Two-Sided Pr > |Z|	0.0903
t Approximation	
One-Sided Pr > Z	0.0555
Two-Sided Pr > |Z|	0.1110

Z includes a continuity correction of 0.5.

Kruskal-Wallis Test

Chi-Square	3.0504
DF	1
Pr > Chi-Square	0.0807

Median Scores (Number of Points Above Median) for Variable CI
Classified by Variable site

site	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
BG	9	3.0	4.50	1.024695	0.333333
CP	7	5.0	3.50	1.024695	0.714286

Median Two-Sample Test

Statistic	5.0000
Z	1.4639
One-Sided Pr > Z	0.0716
Two-Sided Pr > |Z|	0.1432

Median One-Way Analysis

Chi-Square	2.1429
DF	1
Pr > Chi-Square	0.1432

NONPARAMETRIC COMPARISON OF CONDITION INDEX (CI) BETWEEN SITES 90

-- Sex=M --

The NPAR1WAY Procedure

Wilcoxon Scores (Rank Sums) for Variable CI
Classified by Variable site

site	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
BG	22	349.0	594.0	55.398556	15.863636
CP	11	305.0	297.0	45.596052	27.727273
UM	20	777.0	540.0	54.497706	38.850000

Kruskal-Wallis Test

Chi-Square	23.2397
DF	2
Pr > Chi-Square	<.0001

Median Scores (Number of Points Above Median) for Variable CI
Classified by Variable site

site	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
BG	22	1.0	10.792453	1.810436	0.045455
CP	11	7.0	5.396226	1.490088	0.636364
UM	20	18.0	9.811321	1.780996	0.900000

Median One-Way Analysis

Chi-Square	31.1926
DF	2
Pr > Chi-Square	<.0001

ANOVA FOR E2/T RATIO BETWEEN SITES 92

--- Sex=F ---

The ANOVA Procedure

Class Level Information

Class	Levels	Values
site	3	BG CP UM

Number of observations 60

NOTE: Due to missing values, only 42 observations can be used in this analysis.

Dependent Variable: RATIO

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	1	21.055872	21.055872	0.15	0.6999
Error	40	5587.442993	139.686075		
Corrected Total	41	5608.498865			

R-Square	Coeff Var	Root MSE	RATIO Mean
0.003754	161.1670	11.81889	7.333315

Source	DF	Anova SS	Mean Square	F Value	Pr > F
site	1	21.05587231	21.05587231	0.15	0.6999

Levene's Test for Homogeneity of RATIO Variance
ANOVA of Squared Deviations from Group Means

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
site	1	53436.8	53436.8	0.32	0.5745
Error	40	6670870	166772		

Bartlett's Test for Homogeneity of RATIO Variance

Source	DF	Chi-Square	Pr > ChiSq
site	1	1.4679	0.2257

Dunnett's t Tests for RATIO

NOTE: This test controls the Type I experimentwise error for comparisons of all treatments against a control.

Alpha	0.05
Error Degrees of Freedom	40
Error Mean Square	139.6861
Critical Value of Dunnett's t	2.02111

Comparisons significant at the 0.05 level are indicated by ***.

site Comparison	Difference Between Means	Simultaneous 95% Confidence Limits	
CP - BG	1.478	-6.215	9.170

-- Sex=I --

The ANOVA Procedure

Class Level Information

Class	Levels	Values
site	2	BG CP

Number of observations 16

NOTE: Due to missing values, only 15 observations can be used in this analysis.

Dependent Variable: RATIO

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	1	3.54538874	3.54538874	0.57	0.4636
Error	13	80.81750386	6.21673107		
Corrected Total	14	84.36289260			

R-Square	Coeff Var	Root MSE	RATIO Mean
0.042025	100.0048	2.493337	2.493217

Source	DF	Anova SS	Mean Square	F Value	Pr > F
site	1	3.54538874	3.54538874	0.57	0.4636

Levene's Test for Homogeneity of RATIO Variance
ANOVA of Squared Deviations from Group Means

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
site	1	7.8877	7.8877	0.13	0.7221
Error	13	776.1	59.6964		

Bartlett's Test for Homogeneity of RATIO Variance

Source	DF	Chi-Square	Pr > ChiSq
site	1	0.0674	0.7951

Dunnett's t Tests for RATIO

NOTE: This test controls the Type I experimentwise error for comparisons of all treatments against a control.

Alpha	0.05
Error Degrees of Freedom	13
Error Mean Square	6.216731
Critical Value of Dunnett's t	2.16042

Comparisons significant at the 0.05 level are indicated by ***.

site Comparison	Difference Between Means	Simultaneous 95% Confidence Limits	
CP - BG	-0.9924	-3.8314	1.8466

ANOVA FOR E2/T RATIO BETWEEN SITES 100

--- Sex=M ---

The ANOVA Procedure

Class Level Information

Class	Levels	Values
site	3	BG CP UM

Number of observations 53

NOTE: Due to missing values, only 42 observations can be used in this analysis.

Dependent Variable: RATIO

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	2	6.0121986	3.0060993	0.88	0.4222
Error	39	132.9691436	3.4094652		
Corrected Total	41	138.9813422			

R-Square	Coeff Var	Root MSE	RATIO Mean
0.043259	132.2498	1.846474	1.396201

Source	DF	Anova SS	Mean Square	F Value	Pr > F
site	2	6.01219864	3.00609932	0.88	0.4222

Levene's Test for Homogeneity of RATIO Variance
ANOVA of Squared Deviations from Group Means

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
site	2	148.8	74.3982	0.47	0.6311
Error	39	6230.2	159.7		

Bartlett's Test for Homogeneity of RATIO Variance

Source	DF	Chi-Square	Pr > ChiSq
site	2	9.7484	0.0076

Dunnett's t Tests for RATIO

NOTE: This test controls the Type I experimentwise error for comparisons of all treatments against a control.

Alpha	0.05
Error Degrees of Freedom	39
Error Mean Square	3.409465
Critical Value of Dunnett's t	2.31338

Comparisons significant at the 0.05 level are indicated by ***.

site Comparison	Difference Between Means	Simultaneous 95% Confidence Limits	
CP - BG	-0.3223	-2.0859	1.4413
UM - BG	-0.8799	-2.4128	0.6531

NONPARAMETRIC COMPARISON OF E2/T RATIO BETWEEN SITES 104

--- Sex=F ---

The NPAR1WAY Procedure

Wilcoxon Scores (Rank Sums) for Variable RATIO
Classified by Variable site

site	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
BG	15	269.0	322.50	38.095275	17.933333
CP	27	634.0	580.50	38.095275	23.481481

Wilcoxon Two-Sample Test

Statistic	269.0000
Normal Approximation	
Z	-1.3912
One-Sided Pr < Z	0.0821
Two-Sided Pr > |Z|	0.1642
t Approximation	
One-Sided Pr < Z	0.0858
Two-Sided Pr > |Z|	0.1717

Z includes a continuity correction of 0.5.

Kruskal-Wallis Test

Chi-Square	1.9723
DF	1
Pr > Chi-Square	0.1602

Median Scores (Number of Points Above Median) for Variable RATIO
Classified by Variable site

site	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
BG	15	5.0	7.50	1.571468	0.333333
CP	27	16.0	13.50	1.571468	0.592593

Median Two-Sample Test

Statistic	5.0000
Z	-1.5909
One-Sided Pr < Z	0.0558
Two-Sided Pr > |Z|	0.1116

Median One-Way Analysis

Chi-Square	2.5309
DF	1
Pr > Chi-Square	0.1116

-- Sex=I --

The NPAR1WAY Procedure

Wilcoxon Scores (Rank Sums) for Variable RATIO
Classified by Variable site

site	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
BG	9	83.0	72.0	8.485281	9.222222
CP	6	37.0	48.0	8.485281	6.166667

Wilcoxon Two-Sample Test

Statistic	37.0000
Normal Approximation	
Z	-1.2374
One-Sided Pr < Z	0.1080
Two-Sided Pr > |Z|	0.2159
t Approximation	
One-Sided Pr < Z	0.1181
Two-Sided Pr > |Z|	0.2363

Z includes a continuity correction of 0.5.

Kruskal-Wallis Test

Chi-Square	1.6806
DF	1
Pr > Chi-Square	0.1949

Median Scores (Number of Points Above Median) for Variable RATIO
Classified by Variable site

site	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
BG	9	5.0	4.20	0.979796	0.555556
CP	6	2.0	2.80	0.979796	0.333333

Median Two-Sample Test

Statistic	2.0000
Z	-0.8165
One-Sided Pr < Z	0.2071
Two-Sided Pr > |Z|	0.4142

Median One-Way Analysis

Chi-Square	0.6667
DF	1
Pr > Chi-Square	0.4142

--- Sex=M ---

The NPAR1WAY Procedure

Wilcoxon Scores (Rank Sums) for Variable RATIO
Classified by Variable site

site	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
BG	22	542.0	473.0	39.707262	24.636364
CP	8	157.0	172.0	31.219652	19.625000
UM	12	204.0	258.0	35.916570	17.000000

Kruskal-Wallis Test

Chi-Square	3.2394
DF	2
Pr > Chi-Square	0.1980

Median Scores (Number of Points Above Median) for Variable RATIO
Classified by Variable site

site	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
BG	22	14.0	11.0	1.637964	0.636364
CP	8	4.0	4.0	1.287842	0.500000
UM	12	3.0	6.0	1.481594	0.250000

Median One-Way Analysis

Chi-Square	4.5260
DF	2
Pr > Chi-Square	0.1040

--- Sex=F ---

The ANOVA Procedure

Class Level Information

Class	Levels	Values
site	3	BG CP UM

Number of observations 60

NOTE: Due to missing values, only 20 observations can be used in this analysis.

Dependent Variable: P_mg

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	2	11649.5373	5824.7687	0.32	0.7294
Error	17	308087.4127	18122.7890		
Corrected Total	19	319736.9500			

R-Square	Coeff Var	Root MSE	P_mg Mean
0.036435	15.97021	134.6209	842.9500

Source	DF	Anova SS	Mean Square	F Value	Pr > F
site	2	11649.53730	5824.76865	0.32	0.7294

Levene's Test for Homogeneity of P_mg Variance
ANOVA of Squared Deviations from Group Means

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
site	2	33916350	16958175	0.05	0.9474
Error	17	5.3185E9	3.1285E8		

Bartlett's Test for Homogeneity of P_mg Variance

Source	DF	Chi-Square	Pr > ChiSq
site	2	0.0751	0.9631

Dunnett's t Tests for P_mg

NOTE: This test controls the Type I experimentwise error for comparisons of all treatments against a control.

Alpha	0.05
Error Degrees of Freedom	17
Error Mean Square	18122.79
Critical Value of Dunnett's t	2.42696

Comparisons significant at the 0.05 level are indicated by ***.

site Comparison	Difference Between Means	Simultaneous 95% Confidence Limits	
CP - BG	54.37	-110.29	219.02
UM - BG	25.72	-170.61	222.06

--- Sex=I ---

The ANOVA Procedure

Class Level Information

Class	Levels	Values
site	2	BG CP

Number of observations 16

NOTE: Due to missing values, only 14 observations can be used in this analysis.
Dependent Variable: P_mg

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	1	532.1488	532.1488	0.04	0.8535
Error	12	179378.2083	14948.1840		
Corrected Total	13	179910.3571			

R-Square	Coeff Var	Root MSE	P_mg Mean
0.002958	15.80060	122.2628	773.7857

Source	DF	Anova SS	Mean Square	F Value	Pr > F
site	1	532.1488095	532.1488095	0.04	0.8535

Levene's Test for Homogeneity of P_mg Variance
ANOVA of Squared Deviations from Group Means

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
site	1	75518806	75518806	0.30	0.5920
Error	12	2.9897E9	2.4914E8		

Bartlett's Test for Homogeneity of P_mg Variance

Source	DF	Chi-Square	Pr > ChiSq
site	1	0.1445	0.7038

Dunnett's t Tests for P_mg

NOTE: This test controls the Type I experimentwise error for comparisons of all treatments against a control.

Alpha	0.05
Error Degrees of Freedom	12
Error Mean Square	14948.18
Critical Value of Dunnett's t	2.17886

Comparisons significant at the 0.05 level are indicated by ***.

site Comparison	Difference Between Means	Simultaneous 95% Confidence Limits	
CP - BG	-12.46	-156.33	131.41

ANOVA FOR VITELLOGENIN (P_mg) BETWEEN SITES 118

--- Sex=M ---

The ANOVA Procedure

Class Level Information

Class	Levels	Values
site	3	BG CP UM

Number of observations 53

NOTE: Due to missing values, only 24 observations can be used in this analysis.

Dependent Variable: P_mg

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	2	182143.0833	91071.5417	8.25	0.0023
Error	21	231848.7500	11040.4167		
Corrected Total	23	413991.8333			

R-Square	Coeff Var	Root MSE	P_mg Mean
0.439968	27.98847	105.0734	375.4167

Source	DF	Anova SS	Mean Square	F Value	Pr > F
site	2	182143.0833	91071.5417	8.25	0.0023

Levene's Test for Homogeneity of P_mg Variance
ANOVA of Squared Deviations from Group Means

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
site	2	5.3083E8	2.6541E8	0.75	0.4831
Error	21	7.399E9	3.5233E8		

Bartlett's Test for Homogeneity of P_mg Variance

Source	DF	Chi-Square	Pr > ChiSq
site	2	2.8489	0.2406

Dunnett's t Tests for P_mg

NOTE: This test controls the Type I experimentwise error for comparisons of all treatments against a control.

Alpha	0.05
Error Degrees of Freedom	21
Error Mean Square	11040.42
Critical Value of Dunnett's t	2.37033
Minimum Significant Difference	124.53

Comparisons significant at the 0.05 level are indicated by ***.

site Comparison	Difference Between Means	Simultaneous 95% Confidence Limits		
CP - BG	51.63	-72.90	176.15	
UM - BG	-153.50	-278.03	-28.97	***

NONPARAMETRIC COMPARISON OF VITELLOGENIN (P_mg) BETWEEN SITES 122

-- Sex=F --

The NPAR1WAY Procedure

Wilcoxon Scores (Rank Sums) for Variable P_mg
Classified by Variable site

site	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
BG	9	84.0	94.50	13.162447	9.333333
CP	7	81.0	73.50	12.619429	11.571429
UM	4	45.0	42.00	10.583005	11.250000

Kruskal-Wallis Test

Chi-Square	0.6439
DF	2
Pr > Chi-Square	0.7247

Median Scores (Number of Points Above Median) for Variable P_mg
Classified by Variable site

site	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
BG	9	4.0	4.50	1.141329	0.444444
CP	7	4.0	3.50	1.094243	0.571429
UM	4	2.0	2.00	0.917663	0.500000

Median One-Way Analysis

Chi-Square	0.2413
DF	2
Pr > Chi-Square	0.8864

NONPARAMETRIC COMPARISON OF VITELLOGENIN (P_mg) BETWEEN SITES 124

--- Sex=I ---

The NPAR1WAY Procedure

Wilcoxon Scores (Rank Sums) for Variable P_mg
Classified by Variable site

site	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
BG	8	61.0	60.0	7.745967	7.625000
CP	6	44.0	45.0	7.745967	7.333333

Wilcoxon Two-Sample Test

Statistic	44.0000
Normal Approximation	
Z	-0.0645
One-Sided Pr < Z	0.4743
Two-Sided Pr > |Z|	0.9485
t Approximation	
One-Sided Pr < Z	0.4748
Two-Sided Pr > |Z|	0.9495

Z includes a continuity correction of 0.5.

Kruskal-Wallis Test

Chi-Square	0.0167
DF	1
Pr > Chi-Square	0.8973

Median Scores (Number of Points Above Median) for Variable P_mg
Classified by Variable site

site	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
BG	8	4.0	4.0	0.960769	0.50
CP	6	3.0	3.0	0.960769	0.50

Median Two-Sample Test

Statistic	3.0000
Z	0.0000
One-Sided Pr < Z	0.5000
Two-Sided Pr > |Z|	1.0000

Median One-Way Analysis

Chi-Square	0.0000
DF	1
Pr > Chi-Square	1.0000

NONPARAMETRIC COMPARISON OF VITELLOGENIN (P_mg) BETWEEN SITES 126

--- Sex=M ---

The NPAR1WAY Procedure

Wilcoxon Scores (Rank Sums) for Variable P_mg
Classified by Variable site

site	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
BG	8	117.0	100.0	16.329932	14.6250
CP	8	138.0	100.0	16.329932	17.2500
UM	8	45.0	100.0	16.329932	5.6250

Kruskal-Wallis Test

Chi-Square	11.8950
DF	2
Pr > Chi-Square	0.0026

The NPAR1WAY Procedure

Median Scores (Number of Points Above Median) for Variable P_mg
Classified by Variable site

site	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
BG	8	5.0	4.0	1.179536	0.6250
CP	8	7.0	4.0	1.179536	0.8750
UM	8	0.0	4.0	1.179536	0.0000

Median One-Way Analysis

Chi-Square	12.4583
DF	2
Pr > Chi-Square	0.0020

Data Evaluation Report on Response of Larval *Xenopus laevis* to Atrazine Exposure: Assessment of Metamorphosis and Gonadal and Laryngeal Morphology

EPA MRID Number 458677-07

Data Requirement: :

EPA DP Barcode D288775

EPA MRID 458677-07
EPA Guideline 70-1(Special Study)

Test material: **Purity:** 98.6%

Common name Atrazine
Chemical name: IUPAC
CAS name 6-chloro-N-ethyl-N'-(1-methylethyl)-1,3,5-triazine-2,4-diamine
CAS No. 1912-24-9
Synonyms
EPA PC Code: 80803

Thomas M Steeger

Primary Reviewer: Thomas M. Steeger, Ph.D., Senior Biologist **Date:** April 6, 2003
Environmental Fate and Effects Division, ERB 4,
U. S. Environmental Protection Agency

Joseph E. Tietge

Secondary Reviewer(s): Joseph E. Tietge, M.S., Research Aquatic Biologist **Date:** 4/16/03
Mid-Continent Ecology Division, National Health and Environmental Effects Research Laboratory (Duluth), U. S. Environmental Protection Agency

Stephanie R. Irene

Stephanie Irene, Ph.D., Senior Advisor **Date:** 5/6/03
Environmental Fate and Effects Division, ERB 3, U. S. Environmental Protection Agency

Mary J. Frankenberry, Senior Statistician **Date:** 5/05/03
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CITATION: Goleman, W. L. and J. A. Carr. 2003. Response of larval *Xenopus laevis* to Atrazine Exposure: Assessment of Metamorphosis and Gonadal and Laryngeal Morphology. The Institute of Environmental and Human Health, Texas Tech University, Texas Tech University Health Sciences Center, Lubbock, Texas. Sponsor: Syngenta Crop Protection, Inc., Laboratory Study ID ECORISK Number TTU-01.

EXECUTIVE SUMMARY:

In a 78-day exposure, 48- to 72-hr post-hatch African clawed frog (*Xenopus laevis*) larvae were exposed to nominal concentrations of 1, 10 and 25 μg atrazine/L in FETAX medium, FETAX medium alone (negative control), 17-β estradiol, dihydrotestosterone, or solvent control (0.0025% ethanol) in a static renewal system where 50% exposure solution water changes occurred every 72 hours. For the first seven days, 60 - 65 larvae were maintained in 100 mL of exposure solution. On day 14, animals were transferred to 1 L of exposure solution, and by Day 21, animals were maintained in 4 L of exposure solution. At NF Stage 66 (forelimb emergence and complete tail resorption) animals were weighed, measured for snout-vent length and examined for gonadal gross morphology. Larynx and gonads also underwent histological analysis.

Mortality over the study period ranged from 10 to 14% for those animals that reached stage 66 by 80 days post-hatch. Time to complete metamorphosis was not significantly different across treatments although the specific time was not reported. In all treatments, weight and snout-vent length were inversely proportional to the number of days required to complete metamorphosis, *i.e.*, animals completing metamorphosis early tended to be larger than animals that took longer to complete metamorphosis. Sex ratios ranged from 48% to 50% male across all treatments except for estradiol, which skewed the ratio in favor of females (67%). While the incidence of intersex was correlated with atrazine treatment concentrations, only the 25 μg atrazine/L (4.7%) and estradiol-treated (7.4%) males had incidence rates significantly different from negative (0.6%) and solvent (0.0%) controls. Intersex in males treated with 25 μg atrazine/L contained distinguishable testicular and ovarian tissue, while males treated with estradiol sometimes contained ambiguous tissue structures.

There was no difference in the cross-sectional area of larynx dilator muscle in any atrazine-treated males relative to negative controls. Dihydrotestosterone-treated females had significantly larger cross-sectional dilator muscle areas than solvent control females.

Although the report concluded that atrazine did not impact length, weight, time to metamorphosis or dilator muscle area relative to controls, it did conclude that exposure to 25 μg atrazine/L appeared to significantly increase the number of intersex males and animals with discontinuous gonads. However, the observation that both body weight and length were inversely correlated with the length of time to complete metamorphosis suggested that animals in all treatment groups were developmentally impaired. Also, the fact that 17-β estradiol treatment only resulted in 67% females, further suggested that study animals were not entirely responsive to the positive control. Although dissolved oxygen did not appear to drop below 3.9 mg/L, ammonia levels ranged as high as 27 mg/L suggesting that the 50% static renewal and loading rates (number of tadpoles per liter of exposure solution) may have resulted in poor water quality that could, in turn, impair the development of test animals. Furthermore, roughly 42% of the animals were assumed not to have reached stage 66 by Day 78, suggesting that a large proportion of the animals were not developing at all. Because of the declining condition of the frogs with increased length of time to maturity, it is uncertain whether these animals completed metamorphosis and/or survived. Furthermore, because all the animals in the study had not undergone metamorphosis, the percent initiating metamorphosis, time to metamorphosis and percentage of gonadal abnormalities could not be accurately calculated relative to the total animals used in the study.

This study indicates that only one exposure level (25 ug/L) produced developmental effects in frogs, but because of design problems the study did not establish a dose-response relationship between atrazine exposure and developmental effects in frogs. The ability of this study to serve as a sensitive indicator of developmental effects is also in question because of the impaired condition of the animals.

I. MATERIALS AND METHODS

GUIDELINE FOLLOWED: Nonguideline Study

COMPLIANCE: Not conducted under full Good Laboratory Practices; however, most of the practices as defined by 40 CFR Part 160, August 19, 1989, were established for this study, including but not limited to:

- Written, authorized protocol
- Written, authorized Standard Operating Procedures for all key procedures.
- Organization and Personnel were sufficient in terms of number, education, training and experience.
- Facilities were of suitable size and construction
- Equipment used was of appropriate design and adequate capacity.
- Test material identity, strength, purity and composition were characterized.
- Independent QA Inspections were conducted.
- Final Report was written
- Raw data, documentation, records, protocols, and final report were archieved.

A. MATERIALS:

1. Test Material Atrazine

Description: Not reported

Lot No./Batch No. : Not reported

Purity: 98.6%
Stability of compound under test conditions: Not reported

Storage conditions of test chemicals: Not reported

2. Test organism:

Species: African clawed frog (*Xenopus laevis*)

Age at test initiation: 48 - 72 hr larvae
Weight at study initiation: (mean and range) Not reported
Length at study initiation: (mean and range) Not reported

Source: Six breeding pairs of *X. laevis* obtained from Xenopus Express (Homosassa, FL) artificially induced to spawn.

B. STUDY DESIGN:

Objective: 1. To determine the response of larval *Xenopus laevis* to atrazine by assessing the metamorphosis and reproductive indices when animals are exposed from 48 - 72 hours after hatching until the completion of metamorphosis. Indices to be evaluated include percent initiating metamorphosis, percent completing metamorphosis, time to metamorphosis, percentage of intersex gonads, fresh post-mortem body weight, snout-vent length, and laryngeal size.

1. Experimental Conditions

a) **Range-finding Study**: Trials 1 - 3 were conducted in 0.1x Holtfreter's solution and were terminated due to unexpectedly high mortality in all concentrations. In Trial 1, larvae were transferred 48 hr post-hatch and all the transferred animals died within 24 hours of transfer from "damaged abdomens". In Trials 2 and 3, exposures began when larvae were less than 24 hrs old resulting in "unacceptably high mortality" and poor growth leaving study authors to conclude that 0.1x Holtfretter's was unsuitable as a medium for raising larvae. FETAX medium was used in the fourth and final attempt at a definitive study.

b) **Definitive Study**

Table 1 . Experimental Parameters

Parameter	Details
Acclimation: period: Conditions: (same as test or not) Feeding: Health: (any mortality observed)	breeding adults maintained separately for 7 days before breeding; maintained in 45-L glass tanks containing 18-L of ultra-pure water at 22 ± 1°C. Beginning 4 days prior to breeding, adult tank water changed to FETAX by daily 50% medium changes.
Duration of the test	78 days or until frogs reached NF stage 66 (forelimb emergence and complete tail resorption) of development
Test condition static/flow- through Type of dilution system for flow-through method. Renewal rate for static renewal	 static renewal NA 50% exposure solution change every 72 hours
Aeration, if any	not reported

Parameter	Details
Test vessel Material: (glass/stainless steel) Size: Fill volume:	 Glass beaker Initially 250 mL; on Day 7 transferred to 2-L beakers; on Day 21 transferred to 10-L beakers Initially 100 mL; on Day 7 filled with 1-L; on Day 21 filled with 4 L
Source of dilution water Quality:	tap water run through reverse osmosis and deionizer to convert to ultra-pure water
Water parameters: Hardness pH Dissolved oxygen Total Organic carbon Particulate Matter Ammonia Nitrite Metals Pesticides Chlorine Temperature Salinity Intervals of water quality measurement	 not reported range: 6.9 to 8.3 range: 3.9 to 9.3 mg/L; mean 6.7 mg/L not reported not reported range: 0 to 27.1 mg/L (unionized 0 - 0.24 mg/L); mean 9.4 mg/L not reported not reported not reported not reported range: 19 to 22.8°C range: 0.6 to 1.2 ppt temperature monitored daily; pH, conductivity, salinity, DO and ammonia monitored on freshly prepared stock solutions and 72-hr exposure solutions (every 72 hours).
Number of replicates/groups: negative control: water treated ones:	11 replicates for atrazine and negative control; 6 replicates for ethanol, dihydrotestosterone and 17-β estradiol (0.1 μg/mL) positive controls.
Number of organisms per replicate /groups:	60 - 65 larvae per replicate
Biomass loading rate	Initially, 650/L, at Day 7 65/L and at Day 21 onward 16.3/ L

Parameter	Details
Test concentrations: nominal: measured:	 0, 1, 10 and 25 µg/L 1.07 ± 0.02 µg/L, 10.3 ± 0.15 µg/L, 19.5 ± 0.21 µg/L
Solvent (type, percentage, if used)	FETAX solution for atrazine treated and negative control steroids and solvent control in 0.0025% ethanol/FETAX. FETAX medium: NaCl 0.625 g/L; $NaHCO_3$ 0.096 g/L; KCl 0.03 g/L; $CaCl_2$ 0.015 g/L, $CaSO_4$-$2H_2O$ 0.06 g/L; $MgSO_4$ 0.075 g/L
Lighting	12 hrs light, 12 hrs dark
Feeding	not reported
Recovery of chemical Level of Quantitation Level of Detection	Measured using ELISA (RAPID Strategic Diagnostics, Newark, DE); approximately 10% of samples verified by second analysis.
Positive control {if used, indicate the chemical and concentrations}	dihydrotestosterone (0.1 µg/L in 0.0025% ethanol) 17-β estradiol (0.1 µg/L in 0.0025% ethanol)
Other parameters, if any	

2. Observations:

Table 2: Observations

Criteria	Details
Parameters measured including the sublethal effects/toxicity symptoms	water quality (excess food, etc.); % mortality, % showing forelimb emergence, % metamorphosed (complete tail resorption)
Observation intervals	monitored daily for changes in general health and for parameters described above.
Were raw data included?	
Other observations, if any	

II. RESULTS and DISCUSSION:

Atrazine concentrations in both the freshly prepared stock solutions and the exposure tanks were relatively consistent with nominal values (**Table 3**).

Table 3. Nominal and mean-measured atrazine concentrations in freshly prepared stock solutions and exposure tanks.

Nominal	Stock Solution	Exposure Tank
1 µg/L	1.02 ± 0.03 µg/L	1.07 ± 0.02 µg/L
10 µg/L	9.99 ± 0.29 µg/L	10.3 ± 0.15 µg/L
25 µg/L	21.3 ± 0.67 µg/L	19.5 ± 0.21 µg/L

Estradiol concentrations in exposure tanks A through F ranged from 5,520 pg/mL to 11,700 pg/mL (mean: 9,255 pg/mL) on August 21. On October 5, 2001, estradiol levels ranged from 4,764 to 17,467 pg/mL (mean: 9,897 pg/mL). Therefore, average estradiol concentrations were 0.009 µg/L and were roughly 9% of the desired nominal estradiol concentration of 0.1%.

Post-hatch mortality across all study groups ranged from 10 to 14% (**Table 4**). There was no difference in post-hatch mortality for atrazine treatments and the negative control (ANOVA, $p = 0.2$) or for hormone treatments and the ethanol control (ANOVA, $p = 0.7$). There was no difference in body weight or snout-vent length among atrazine-treated and negative controls. Time to complete metamorphosis (NF stage 66) varied inversely with body weight and SVL; those animals reaching NF-stage 66 first in each tank were significantly larger than those animals that were the last, *i.e.*, animals reaching NF-stage 66 by Day 78, in every treatment (paired t-test, $p<0.05$). Based on the report figure depicting body weights, the average weight of animals reaching stage 66 first ranged from 0.48 to 0.62 g, while the last animals to reach stage 66 had body weights ranging from 0.26 to 0.30 g. Average snout-vent length for animals reaching stage 66 first ranged from 16 to 17 mm while lengths for animals reaching stage 66 last ranged from 13 to 13.5 mm roughly. Body weight and SVL were also inversely correlated with time to complete metamorphosis in hormone-treated and solvent controls. Body weights for animals reaching stage 66 first in hormone and solvent-treated animals ranged from 0.48 to 0.54 g, while late stage 66 animals ranged from 0.24 to 0.25 g. Estradiol-treated females had significantly longer SVL than solvent controls (ANOVA, $p = 0.02$).

The percentage of animals reaching complete tail resorption varied between 37 - 52% during the 78-day period. Fewer atrazine-treated (referred to as a "weak trend" by authors) animals reached (ANOVA trend test, $p = 0.03$) and completed tail resorption (ANOVA trend test, $p = 0.04$). However, the general ANOVA F-test revealed no significant effect of atrazine on either parameter compared to negative controls.

The incidence of gross gonadal deformities was less than 5% in all treatments. The incidence of edema was correlated with atrazine concentration (Cochran-Armitage trend test, $Z = -2.3$, $p=0.02$); however, a chi-square homogeneity test revealed that none of the atrazine concentrations significantly increased the incidence of edema compared to negative controls ($p = 0.1$). Abnormal swimming was also correlated with atrazine concentration (Cochran-Armitage trend test, $Z = -2.90$, $p = 0.004$), but only larvae exposed to 25 µg atrazine/L exhibited significantly greater incidence of abnormal swimming (chi-square homogeneity test, $p = 0.04$).

There were no detectable effects on sex ratio with ratios ranging from 48 to 50% males in all treatments except the 25 µg atrazine/L treatment. There was a slight reduction in percentage (45%) of males in the 25 µg atrazine/L. Neither ethanol nor dihyrotestosterone significantly affected sex ratios. Estradiol significantly reduced the percentage of males to 26% and increased the percentage of females to 67%. Approximately 7% of the estradiol-treated animals were classified as intersex based on gross morphology.

Exposure to either 25 µg atrazine/L or estradiol significantly increased the percentage of individuals with intersex gonads. Although the incidence of intersex increased with increasing atrazine concentrations (Cochran-Armitage, Z= 3.6, $p = 0.0003$), only 25 µg atrazine/L significantly increased the average incidence of intersex animals (4.7%) per tank compared to negative control (0.6%) ($p = 0.0061$) (**Table 5**). There was also a significant correlation between the incidence of discontinuous gonads and atrazine concentration (Cochran-Armitage Z = 2.9, $p = 0.0042$) although 25 µg atrazine/L was the only concentration to increase the incidence of discontinuous gonads compared to negative controls. The percentage of intersex gonads was also significantly higher in the estradiol-treated group compared to the solvent control. (Kruskall-Wallis, $p = 0.01$).

Table 5. Total number of frogs identified as intersex by treatment.

Treatment	Total Animals	Intersex	Percentage
0	334	2	0.6%
1 µg atrazine/L	309	3	0.97%
10 µg atrazine/L	276	1	0.36%
25 µg atrazine/L	296	14	4.7%
estradiol	135	10	7.4%
solvent control	160	0	0.0%

Intersex animals in the 25 µg atrazine/L group tended to have obvious testicular or ovarian tissues, while estradiol-treated animals revealed "ambiguous" gonadal tissue in some cases.

There were no significant differences in male dilator muscle size within any of the negative control or atrazine treatments (**Table 6**). Total muscle cross-sectional area was 20 - 25% larger in males than females in all atrazine-treated groups. There were no significant effects of atrazine on total cross-sectional area in females ($p = 0.5$). Exposure to dihydrotestosterone increased total muscle cross-sectional area approximately 2-fold in both males ($p > 0.0001$) and females ($p < 0.0001$). Total muscle cross-sectional area was larger in males than in females in the solvent control group but not in the estradiol and DHT-treated groups.

Table 6. Mean (± standard error) of total dilator muscle cross-sectional area (mm^2) in Nieuwkoop Faber stage 66 *Xenopus laevis* exposed to atrazine or sex steroids[a].

Treatment	Males	Females
negative FETAX control b	0.154 ± 0.004 (n = 45)	0.117 ±0.006 (n = 11)
1 µg atrazine/L	0.167 ± 0.005 (n = 41)	0.118 ± 0.006 (n = 10)
10 µg atrazine/L	0.168 ± 0.004 (n = 44)	0.129 ± 0.007 (n = 11)
25 µg atrazine/L	0.168 ± 0.004 (n = 42)	0.122 ± 0.006 (n = 12)
solvent control (0.0025% ethanol)	0.161 ± 0.008 (n = 6)	0.121 ± 0.013 (n = 6)
dihydrotestosterone	0.301 ±0.012* (n = 6)	0.313 ± 0.024* (n = 6)
17-β estradiol	0.148 ± 0.010 (n = 6)	0.141 ± 0.009 (n = 6)

[a] Combined largest cross-sectional area through the right and left laryngeal dilator muscles.
[b] FETAX: frog embryo teratogenesis assay– *Xenopus*.
* significantly grater than ethanol control (p <0.0001)

F. REVIEWER'S COMMENTS:

For several days, breeding adults were apparently maintained in ultra-pure water before transitioning them into FETAX.

Although samples were collected at the beginning, middle and end of the study for steroid analyses, the report also states though that estradiol was measured using RIA kit (Diagnostic Products Corporation, Los Angeles, CA; LOD 20 pg/mL). DHT values, though, are reported as nominal, and it is unclear whether any effort was made to measure DHT. Measured estradiol concentrations were 0.009 µg/L and were roughly 9% of nominal. Coefficients of variation for the means ranged from 46.5 to 68.9%.

Water samples were collected of freshly prepared stock solutions and of exposure solutions just prior to renewal.

Although the study starts with 60 to 65 larvae per replicate with 11 replicates in the atrazine-treated groups, the results are expressed in terms of 20 - 30 larvae per replicate. If mortality ranged from 10 to 14%, it would leave roughly 52 larvae per replicate, and 42% of the animals would be unaccounted.. It is assumed that the remaining animals failed to reach metamorphosis by Day 78.

The feeding regime was not discussed; however, the report states that frog brittle feed was analyzed by immunoassay and "yielded inconclusive results". Because immunoassays are specific to the antigen of interest, it is presumed that the immunoassay was for atrazine and that something, presumably atrazine, gave variable results, *i.e.*, "yielded inconclusive results." The report states that since a GC/MS method for analyzing frog brittle has not been developed, no data on the food were available (reported).

Body weights of metamorphs were negatively correlated with the length of time to metamorphosis; the number of intersex animals was positively correlated with atrazine concentration.

Although not discussed in the results section, the number of discontinuous gonads appeared to be positively correlated with atrazine treatment. Extrapolating from the figure depicting the percentage of discontinuous gonads there appeared to be 3.5% in controls, and 4%, 7% and 8% in 1, 10 and 25 µg atrazine/L. The number of discontinuous gonads was significantly higher than controls in the 25 µg atrazine/L treatment (chi-square, $p < 0.05$)

The study concluded that estradiol treatment reduced the number of phenotypic males to 25%; however, previous literature suggested that estradiol treatment should result in 100% females.

Body weights and time to metamorphosis were not reported in the study nor was the feeding regime mentioned. Body weights and lengths were extrapolated from figures.

Although the report concluded that atrazine did not impact length, weight, time to metamorphosis or dilator muscle area relative to controls, it did conclude that exposure to 25 µg atrazine/L appeared to significantly increase the number of intersex males and animals with discontinuous gonads. However, the observation that both body weight and length were inversely correlated with the length of time to complete metamorphosis suggested that animals in all treatment groups were developmentally impaired. In addition, 17-β estradiol treatment only resulted in 67% females, which further suggested that study animals were not entirely responsive to the positive control. Although dissolved oxygen did not appear to drop below 3.9 mg/L, ammonia levels did range as high as 27 mg/L, suggesting that the 50% static renewal and loading rates (number of tadpoles per liter of exposure solution) may have resulted in poor water quality, which in turn, accounted for the slow development of test animals.

Roughly 42% of the animals were assumed not to have reached stage 66 by Day 78, suggesting that a large proportion of the animals were not developing at all. Given the declining condition of the frogs with increased length of time to maturity, it is uncertain whether these animals would have undergone metamorphosis and/or survived. Furthermore, since all the animals in the study had not undergone metamorphosis, the percent initiating metamorphosis, time to metamorphosis and percentage of gonadal abnormalities could not be accurately calculated relative to the total animals used in the study.

After 80 days, only 49-61% of the organisms reached forelimb emergence (stage 58) and only 37-52% completed metamorphosis (stage 66). According to Nieuwkoop and Faber, the nominal days to stages 58 and 66 are approximately 44 and 58 days, respectively. Although this can vary between laboratories and conditions, the developmental rates observed in this study are exceedingly slow. (At the National Health and Environmental Effects Research Laboratory researchers typically observe nearly 100% tail resorption in approximately 50-54 days.) Furthermore, there is considerable divergence in development because at least 51-39% of the tested organisms are at or below stage 57, while some have completed metamorphosis.

Taken together, these observations suggest that the organisms were in poor condition. It is unclear, though, how such retarded development could affect sexual differentiation.

The report states that there is no evidence that androgens affected gonadal development in *X. laevis*. While it is apparently true that testosterone is incapable of causing masculinization, DHT, the positive androgen control used in this study, has been shown to masculinize *X. laevis*.

Because only about 50% of the tested organisms reached stage 66, and all of the analyses were conducted on stage 66 organisms, the sampling strategy was probably biased and did not represent the population in the test.

The percentage of tadpoles with abnormal swimming was positively correlated ($r=0.41$; $p=0.006$) with atrazine concentration (see attached SAS© [Statistical Analysis System, Release 8.01, Cary, North Carolina] output); the percentage of tadpoles with edema was also positively correlated ($r= 0.40$; $p=0.007$) with atrazine concentration.

The ability of this study to serve as a sensitive indicator of developmental effects is in question given the seemingly impaired condition of the animals.

G. **CONCLUSIONS:** Although the report concluded that atrazine did not impact length, weight, time to metamorphosis or dilator muscle area relative to controls, it did conclude that exposure to 25 µg atrazine/L appeared to significantly increase the number of intersex males and animals with discontinuous gonads. However, the observation that both body weight and length were inversely correlated with the length of time to complete metamorphosis suggested that animals in all treatment groups were developmentally impaired. Also, the fact that 17-β estradiol treatment only resulted in 67% females, further suggested that study animals were not entirely responsive to the positive control. Although dissolved oxygen did not appear to drop below 3.9 mg/L, ammonia levels ranged as high as 27 mg/L suggesting that the 50% static renewal and loading rates (number of tadpoles per liter of exposure solution) may have resulted in poor water quality that could, in turn, impair the development of test animals. Furthermore, roughly 42% of the animals were assumed not to have reached stage 66 by Day 78, suggesting that a large proportion of the animals were not developing at all. Because of the declining condition of the frogs with increased length of time to maturity, it is uncertain whether these animals completed metamorphosis and/or survived. Furthermore, because all the animals in the study had not undergone metamorphosis, the percent initiating metamorphosis, time to metamorphosis and percentage of gonadal abnormalities could not be accurately calculated relative to the total animals used in the study.

This study indicates that only one exposure level (25 ug/L) produced developmental effects in frogs, but because of design problems the study did not establish a dose-response relationship between atrazine exposure and developmental effects in frogs. The ability of this study to serve as a sensitive indicator of developmental effects is also in question because of the impaired condition of the animals.

H. **REFERENCES:**

Nieuwkoop, P. D. and J. Faber. 1967. Normal table of *Xenopus laevis* (Daudin). North-Holland Publishing Company, Amsterdam.

Table 4. Hatching, mortality, metamorphosis, and gross developmental abnormalities in *X. laevis* tadpoles exposed to atrazine or steroid hormones[a].

Treatment	Hatching (%)	Mortality[b] (%)	Forelimb Emergence (%)	Tail Resorption (%)	Bent Tails (%)	Edema (%)	Abnormal Swimming (%)
Negative	94.9 ± 1.02	10.4 ± 1.07	60.8 ± 3.00	51.5 ± 2.91	0.94 ± 0.43	0.15 ± 0.15	1.37 ± 0.44
1 µg/L	93.6 ± 1.12	11.4 ± 1.20	59.0 ± 3.31	48.4 ± 3.00	0.49 ± 0.36	0.30 ± 0.30	2.15 ± 0.53
10 µg/L	94.5 ± 0.093	14.1 ± 0.99	52.2 ± 3.85	42.3 ± 3.42	2.13 ± 0.46	0.44 ± 0.23	3.12 ± 0.68
25 µg/L	96.0 ± 0.82	12.4 ± 1.58	52.5 ± 2.39	44.4 ± 2.14	1.22 ± 0.33	1.04 ± 0.33	3.75 ± 0.64*
ethanol (0.0025%)	93.7 ± 1.47	11.7 ± 1.62	53.8 ± 4.93	46.1 ± 3.84	0.84 ± 0.56	0.57 ± 0.36	1.99 ± 0.73
Dihydrotestosterone	94.6 ± 2.32	12.2 ± 2.84	48.8 ± 5.36	37.2 ± 4.86	0.80 ± 0.36	1.38 ± 0.49	2.98 ± 0.94
17- estradiol	92.9 ± 2.21	15.2 ± 4.16	51.2 ± 1.91	42.8 ± 2.28	0.82 ± 0.37	0.57 ± 0.36	1.69 ± 0.60

[a] Values are the mean ± standard error of eleven (FETAX medium) or six (ethanol co-solvent) replicates. Sample size per replicate ranged from 20 - 30 animals.
[b] 80-day post-hatch mortality.
* significantly different from FETAX medium (negative) control

PERCENT SURVIVAL BY TREATMENT GROUP 236

Obs	TREAT	_TYPE_	_FREQ_	SURVIVE	STD	CV
1	0.0	0	11	89.5720	3.5310	3.9420
2	1.0	0	11	88.5690	3.9837	4.4979
3	10	0	11	85.9008	3.2759	3.8136
4	25	0	11	87.6466	5.2333	5.9709
5	DHT	0	6	87.8023	6.9606	7.9276
6	ETOH	0	6	88.2824	3.9624	4.4884
7	Estr	0	6	84.8416	10.1787	11.9972

PERCENT OF EGGS HATCHED BY TREATMENT GROUP 237

Obs	TREAT	_TYPE_	_FREQ_	HATCHED	STD	CV
1	0.0	0	11	94.9294	3.38528	3.56610
2	1.0	0	11	93.6032	3.70056	3.95346
3	10	0	11	94.5459	3.09986	3.27868
4	25	0	11	95.9701	2.71563	2.82966
5	DHT	0	6	94.5905	5.68238	6.00734
6	ETOH	0	6	93.6631	3.59493	3.83815
7	Estr	0	6	92.8788	5.41676	5.83207

PERCENT OF TADPOLES WITH BENT TAILS BY TREATMENT GROUP 238

Obs	TREAT	_TYPE_	_FREQ_	BENT	STD	CV
1	0.0	0	11	0.93716	1.41694	151.195
2	1.0	0	11	0.48822	1.17785	241.256
3	10	0	11	2.12556	1.52127	71.571
4	25	0	11	1.21942	1.11046	91.064
5	DHT	0	6	0.80232	0.87911	109.570
6	ETOH	0	6	0.83525	1.37177	164.234
7	Estr	0	6	0.82068	0.90009	109.676

PERCENT OF TADPOLES WITH EDEMA BY TREATMENT GROUP 239

Obs	TREAT	_TYPE_	_FREQ_	EDEMA	STD	CV
1	0.0	0	11	0.14903	0.49428	331.662
2	1.0	0	11	0.30311	0.67450	222.524
3	10	0	11	0.43770	0.74986	171.316
4	25	0	11	1.03520	1.08659	104.965
5	DHT	0	6	1.37917	1.21355	87.992
6	ETOH	0	6	0.56644	0.87922	155.219
7	Estr	0	6	0.56984	0.88284	154.928

PERCENT OF TADPOLES WITH ABNORMAL SWIMMING BY TREATMENT GROUP 240

Obs	TREAT	_TYPE_	_FREQ_	SWIM	STD	CV
1	0.0	0	11	1.37132	1.46806	107.055
2	1.0	0	11	2.15034	1.76982	82.304
3	10	0	11	3.11772	2.25729	72.402
4	25	0	11	3.75251	2.13240	56.826
5	DHT	0	6	2.98382	2.30207	77.152
6	ETOH	0	6	1.98706	1.77746	89.452
7	Estr	0	6	1.69367	1.46398	86.438

PERCENT METAMORPHOSIS BY TREATMENT GROUP 241

Obs	TREAT	_TYPE_	_FREQ_	METAMORP	STD	CV
1	0.0	0	11	51.5133	9.6405	18.7146
2	1.0	0	11	48.3625	9.9718	20.6188
3	10	0	11	42.2644	11.3256	26.7971
4	25	0	11	44.3954	7.1060	16.0061
5	DHT	0	6	37.1615	11.8957	32.0109
6	ETOH	0	6	46.0801	9.4053	20.4107
7	Estr	0	6	42.7528	5.5845	13.0622

The NPAR1WAY Procedure

Wilcoxon Scores (Rank Sums) for Variable PMET
Classified by Variable TREAT

TREAT	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
0.0	11	459.00	346.50	54.267424	41.727273
1.0	11	423.50	346.50	54.267424	38.500000
10	11	298.00	346.50	54.267424	27.090909
25	11	312.00	346.50	54.267424	28.363636
DHT	6	114.50	189.00	41.997885	19.083333
ETOH	6	199.00	189.00	41.997885	33.166667
Estr	6	147.00	189.00	41.997885	24.500000

Average scores were used for ties.

Kruskal-Wallis Test

Chi-Square	9.9774
DF	6
Pr > Chi-Square	0.1256

Median Scores (Number of Points Above Median) for Variable PMET
Classified by Variable TREAT

TREAT	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
0.0	11	8.0	5.50	1.516305	0.727273
1.0	11	7.0	5.50	1.516305	0.636364
10	11	5.0	5.50	1.516305	0.454545
25	11	4.0	5.50	1.516305	0.363636
DHT	6	2.0	3.00	1.173477	0.333333
ETOH	6	3.0	3.00	1.173477	0.500000
Estr	6	2.0	3.00	1.173477	0.333333

Average scores were used for ties.

Median One-Way Analysis

Chi-Square	5.2473
DF	6
Pr > Chi-Square	0.5125

NONPARAMETRIC COMPARISON OF PERCENT SURVIVAL ACROSS TREATMENTS 244

The NPAR1WAY Procedure

Wilcoxon Scores (Rank Sums) for Variable SUR
Classified by Variable TREAT

TREAT	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
0.0	11	426.50	346.50	54.251705	38.772727
1.0	11	377.00	346.50	54.251705	34.272727
10	11	240.50	346.50	54.251705	21.863636
25	11	328.50	346.50	54.251705	29.863636
DHT	6	208.00	189.00	41.985719	34.666667
ETOH	6	185.50	189.00	41.985719	30.916667
Estr	6	187.00	189.00	41.985719	31.166667

Average scores were used for ties.
Kruskal-Wallis Test

Chi-Square	5.4728
DF	6
Pr > Chi-Square	0.4848

Median Scores (Number of Points Above Median) for Variable SUR
Classified by Variable TREAT

TREAT	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
0.0	11	7.750	5.50	1.479165	0.704545
1.0	11	6.750	5.50	1.479165	0.613636
10	11	2.750	5.50	1.479165	0.250000
25	11	4.750	5.50	1.479165	0.431818
DHT	6	4.000	3.00	1.144735	0.666667
ETOH	6	2.000	3.00	1.144735	0.333333
Estr	6	3.000	3.00	1.144735	0.500000

Average scores were used for ties.

Median One-Way Analysis

Chi-Square	6.9240
DF	6
Pr > Chi-Square	0.3279

NONPARAMETRIC COMPARISON OF PERCENT OF TADPOLES WITH ABNORMAL SWIMMING ACROSS TREATMENTS 246

The NPAR1WAY Procedure

Wilcoxon Scores (Rank Sums) for Variable SWIM
Classified by Variable TREAT

TREAT	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
0.0	11	243.50	346.50	53.920541	22.136364
1.0	11	321.00	346.50	53.920541	29.181818
10	11	390.00	346.50	53.920541	35.454545
25	11	462.50	346.50	53.920541	42.045455
DHT	6	221.50	189.00	41.729430	36.916667
ETOH	6	150.50	189.00	41.729430	25.083333
Estr	6	164.00	189.00	41.729430	27.333333

Average scores were used for ties.

Kruskal-Wallis Test

Chi-Square	9.1688
DF	6
Pr > Chi-Square	0.1643

Median Scores (Number of Points Above Median) for Variable SWIM
Classified by Variable TREAT

TREAT	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
0.0	11	4.0	5.50	1.516305	0.363636
1.0	11	5.0	5.50	1.516305	0.454545
10	11	5.0	5.50	1.516305	0.454545
25	11	9.0	5.50	1.516305	0.818182
DHT	6	4.0	3.00	1.173477	0.666667
ETOH	6	1.0	3.00	1.173477	0.166667
Estr	6	3.0	3.00	1.173477	0.500000

Average scores were used for ties.

Median One-Way Analysis

Chi-Square	8.6461
DF	6
Pr > Chi-Square	0.1945

NONPARAMETRIC COMPARISON OF PERCENT OF TADPOLES WITH BENT TAILS ACROSS TREATMENTS 248

The NPAR1WAY Procedure

Wilcoxon Scores (Rank Sums) for Variable BENT
Classified by Variable TREAT

TREAT	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
0.0	11	323.50	346.50	50.384440	29.409091
1.0	11	254.00	346.50	50.384440	23.090909
10	11	491.50	346.50	50.384440	44.681818
25	11	374.00	346.50	50.384440	34.000000
DHT	6	161.00	189.00	38.992820	26.833333
ETOH	6	174.00	189.00	38.992820	29.000000
Estr	6	175.00	189.00	38.992820	29.166667

Average scores were used for ties.

Kruskal-Wallis Test

Chi-Square	10.7175
DF	6
Pr > Chi-Square	0.0975

Median Scores (Number of Points Above Median) for Variable BENT
Classified by Variable TREAT

TREAT	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
0.0	11	4.218750	5.50	1.468156	0.383523
1.0	11	2.281250	5.50	1.468156	0.207386
10	11	9.062500	5.50	1.468156	0.823864
25	11	7.125000	5.50	1.468156	0.647727
DHT	6	3.093750	3.00	1.136215	0.515625
ETOH	6	2.125000	3.00	1.136215	0.354167
Estr	6	3.093750	3.00	1.136215	0.515625

Average scores were used for ties.

Median One-Way Analysis

Chi-Square 10.9792
DF 6
Pr > Chi-Square 0.0890

NONPARAMETRIC COMPARISON OF PERCENT OF TADPOLES WITH EDEMA ACROSS TREATMENTS 250

The NPAR1WAY Procedure

Wilcoxon Scores (Rank Sums) for Variable EDEMA
Classified by Variable TREAT

TREAT	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
0.0	11	266.50	346.50	45.035250	24.227273
1.0	11	300.00	346.50	45.035250	27.272727
10	11	314.50	346.50	45.035250	28.590909
25	11	417.00	346.50	45.035250	37.909091
DHT	6	264.50	189.00	34.853050	44.083333
ETOH	6	192.50	189.00	34.853050	32.083333
Estr	6	198.00	189.00	34.853050	33.000000

Average scores were used for ties.

Kruskal-Wallis Test

Chi-Square 10.2116
DF 6
Pr > Chi-Square 0.1160

Median Scores (Number of Points Above Median) for Variable EDEMA
Classified by Variable TREAT

TREAT	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
0.0	11	3.619048	5.50	1.046350	0.329004
1.0	11	4.357143	5.50	1.046350	0.396104
10	11	5.095238	5.50	1.046350	0.463203
25	11	7.309524	5.50	1.046350	0.664502
DHT	6	4.523810	3.00	0.809776	0.753968
ETOH	6	3.047619	3.00	0.809776	0.507937
Estr	6	3.047619	3.00	0.809776	0.507937

Average scores were used for ties.

Median One-Way Analysis

Chi-Square 9.4273
DF 6
Pr > Chi-Square 0.1509

```
                            Plot of METAMORP*CONC.  Symbol used is '*'.

METAMORP 52 ê
            é
            é  *
            é
            é
            é
            é
         50 ê
            é
            é
            é
            é
            é
            é     *
         48 ê
            é
            é
            é
            é
            é
            é
         46 ê
            é
            é
            é
            é
            é
            é                                                                             *
         44 ê
            é
            é
            é
            é
            é
            é                                *
         42 ê
            é
            èââêââêââêââêââêââêââêââêââêââêââêââêââêââêââêââêââêââêââêââêââêââêââêââêââêââêââ
               0  1  2  3  4  5  6  7  8  9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25

                                                  CONC
```

The CORR Procedure

1 With Variables: CONC
1 Variables: PMET

Simple Statistics

Variable	N	Mean	Std Dev	Sum	Minimum	Maximum
CONC	44	9.00000	10.14087	396.00000	0	25.00000
PMET	44	46.63390	9.96659	2052	19.67213	66.66667

Pearson Correlation Coefficients, N = 44
Prob > |r| under H0: Rho=0

	PMET
CONC	-0.24787 0.1047

```
                                Plot of SWIM*CONC.  Symbol used is '*'.

SWIM 4.0  é
          é
          é                                                                             *
          é
          é
          é
      3.5 ê
          é
          é
          é
          é
          é                                *
          é
      3.0 ê
          é
          é
          é
          é
          é
          é
      2.5 ê
          é
          é
          é
          é
          é     *
          é
      2.0 ê
          é
          é
          é
          é
          é
          é
      1.5 ê
          é
          é  *
          é
          é
          é
      1.0 é
          èââêââêââêââêââêââêââêââêââêââêââêââêââêââêââêââêââêââêââêââêââêââêââêââêââêââêââ
             0  1  2  3  4  5  6  7  8  9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25

                                                   CONC
```

CORRELATION ANALYSIS OF PERCENT OF TADPOLES WITH ABNORMAL SWIMMING OVER CONCENTRATION 255

The CORR Procedure

```
1 With Variables:    CONC
1      Variables:    SWIM
```

Simple Statistics

Variable	N	Mean	Std Dev	Sum	Minimum	Maximum
CONC	44	9.00000	10.14087	396.00000	0	25.00000
SWIM	44	2.59797	2.07806	114.31082	0	7.35294

Pearson Correlation Coefficients, N = 44
Prob > |r| under H0: Rho=0

	SWIM
CONC	0.40804 0.0060

```
Plot of EDEMA*CONC.  Symbol used is '*'.
EDEMA 1.2 ê
          é
          é
          é
          é
          é
          é                                                                             *
      1.0 ê
          é
          é
          é
          é
          é
          é
      0.8 ê
          é
          é
          é
          é
          é
          é
      0.6 ê
          é
          é
          é
          é
          é
          é                                *
      0.4 ê
          é
          é
          é     *
          é
          é
          é
      0.2 ê
          é
          é  *
          é
          é
          é
          é
      0.0 ê
          èââêââêââêââêââêââêââêââêââêââêââêââêââêââêââêââêââêââêââêââêââêââêââêââêââêââêâââ
             0  1  2  3  4  5  6  7  8  9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25
                                                  CONC
```

CORRELATION ANALYSIS OF PERCENT OF TADPOLES WITH EDEMA OVER CONCENTRATION 257

The CORR Procedure

1 With Variables:	CONC
1 Variables:	EDEMA

Simple Statistics

Variable	N	Mean	Std Dev	Sum	Minimum	Maximum
CONC	44	9.00000	10.14087	396.00000	0	25.00000
EDEMA	44	0.48126	0.82662	21.17552	0	3.17460

Pearson Correlation Coefficients, N = 44
Prob > |r| under H0: Rho=0

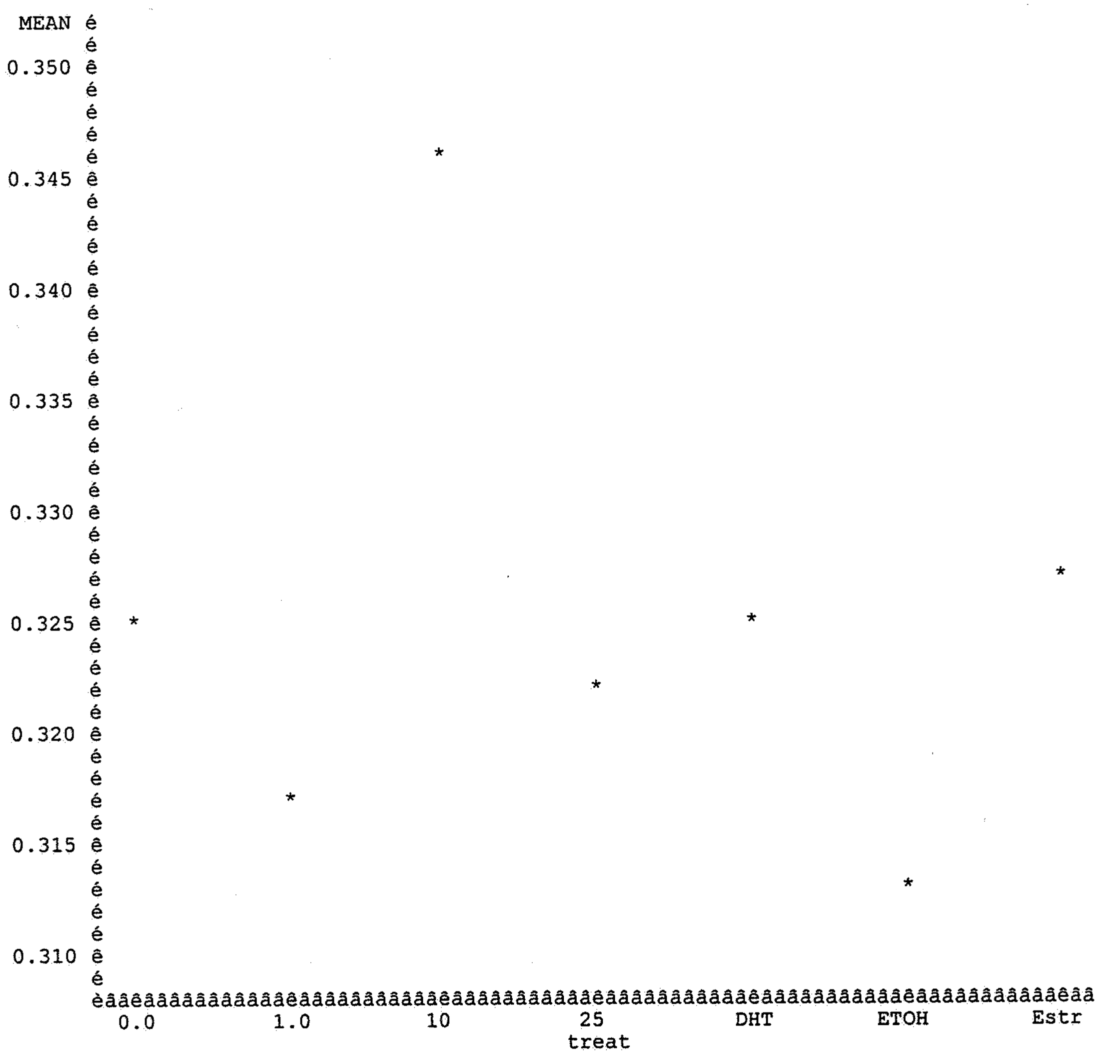
	EDEMA
CONC	0.40388 0.0066

Mean Body Weight of Frogs by Treatment 1202

Obs	treat	_TYPE_	_FREQ_	MEAN	STDERR
1	0.0	0	335	0.32519	.005212036
2	1.0	0	312	0.31670	.005086144
3	10	0	277	0.34574	.006660663
4	25	0	299	0.32173	.005397997
5	DHT	0	135	0.32539	.008838205
6	ETOH	0	161	0.31324	.007248717
7	Estr	0	151	0.32720	.007594965

Mean Body Weight of Frogs by Treatment 1203

Plot of MEAN*treat. Symbol used is '*'.



```
                    Analysis of Variance for Frog Body Weight over Treatment                 1204

                                      The GLM Procedure

                                   Class Level Information

                       Class         Levels    Values

                       treat              7    0.0 1.0 10 25 DHT ETOH Estr


                                Number of observations    1670

NOTE: Due to missing values, only 1661 observations can be used in this analysis.

Dependent Variable: BW

                                              Sum of
      Source                      DF         Squares     Mean Square    F Value    Pr > F

      Model                        6      0.16575046      0.02762508       2.96    0.0071

      Error                     1654     15.45132260      0.00934179

      Corrected Total           1660     15.61707306


                       R-Square     Coeff Var      Root MSE       BW Mean

                       0.010613      29.69766      0.096653      0.325456


      Source                      DF       Type I SS     Mean Square    F Value    Pr > F

      treat                        6      0.16575046      0.02762508       2.96    0.0071


      Source                      DF     Type III SS     Mean Square    F Value    Pr > F

      treat                        6      0.16575046      0.02762508       2.96    0.0071
```

Analysis of Variance for Frog Body Weight over Treatment 1206

The GLM Procedure

Levene's Test for Homogeneity of BW Variance
ANOVA of Squared Deviations from Group Means

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
treat	6	0.00343	0.000571	1.40	0.2098
Error	1654	0.6737	0.000407		

Bartlett's Test for Homogeneity of BW Variance

Source	DF	Chi-Square	Pr > ChiSq
treat	6	17.8554	0.0066

Analysis of Variance for Frog Body Weight over Treatment 1207

The GLM Procedure

Dunnett's t Tests for BW

NOTE: This test controls the Type I experimentwise error for comparisons of all treatments against a control.

Alpha	0.05
Error Degrees of Freedom	1654
Error Mean Square	0.009342
Critical Value of Dunnett's t	2.59779

Comparisons significant at the 0.05 level are indicated by ***.

treat Comparison	Difference Between Means	Simultaneous 95% Confidence Limits		
10 - 0.0	0.020549	0.000124	0.040974	***
Estr - 0.0	0.002005	-0.022617	0.026628	
DHT - 0.0	0.000197	-0.025410	0.025804	
25 - 0.0	-0.003465	-0.023509	0.016578	
1.0 - 0.0	-0.008491	-0.028309	0.011328	
ETOH - 0.0	-0.011958	-0.036098	0.012183	

Nonparametric comparison of frog body weight over treatments 1208

The NPAR1WAY Procedure

Wilcoxon Scores (Rank Sums) for Variable BW
Classified by Variable treat

treat	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
0.0	334	279296.50	277554.0	7834.89949	836.217066
1.0	309	244939.50	256779.0	7606.63049	792.684466
10	276	254956.50	229356.0	7276.19220	923.755435
25	296	242002.00	245976.0	7480.60832	817.574324
DHT	135	109378.50	112185.0	5341.56953	810.211111
ETOH	160	122301.50	132960.0	5767.33359	764.384375
Estr	151	127416.50	125481.0	5619.55184	843.817881

Average scores were used for ties.

Kruskal-Wallis Test

Chi-Square	16.0133
DF	6
Pr > Chi-Square	0.0137

Median Scores (Number of Points Above Median) for Variable BW
Classified by Variable treat

treat	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
0.0	334	178.0	166.899458	8.170047	0.532934
1.0	309	143.0	154.406984	7.932014	0.462783
10	276	162.0	137.916918	7.587441	0.586957
25	296	141.0	147.910897	7.800601	0.476351
DHT	135	61.0	67.459362	5.570062	0.451852
ETOH	160	67.0	79.951836	6.014039	0.418750
Estr	151	78.0	75.454545	5.859935	0.516556

Average scores were used for ties.

Median One-Way Analysis

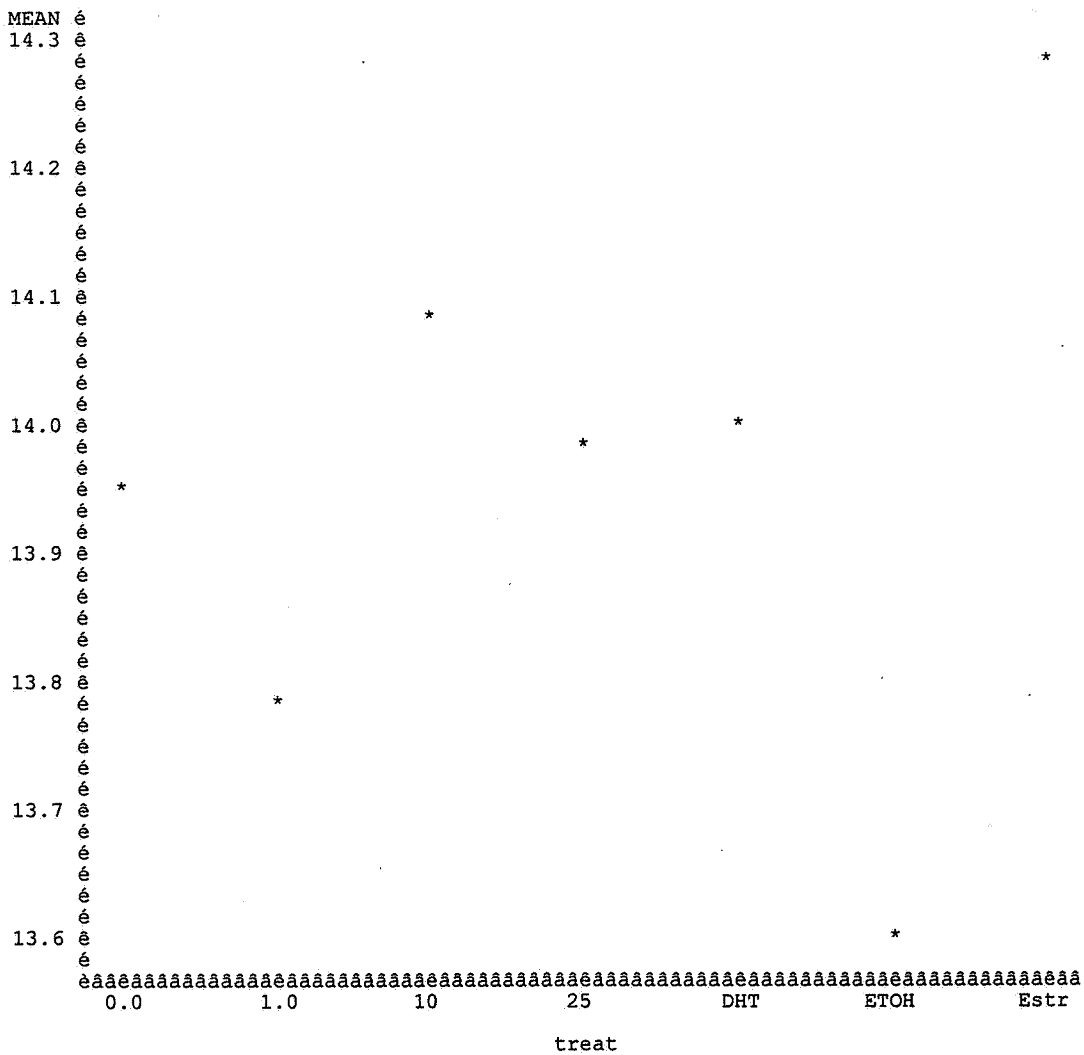
Chi-Square	17.8022
DF	6
Pr > Chi-Square	0.0067

Average Snout to Vent Length of Frogs by Treatment 1210

Obs	treat	_TYPE_	_FREQ_	MEAN	STDERR
1	0.0	0	335	13.9566	0.08149
2	1.0	0	312	13.7896	0.07939
3	10	0	277	14.0815	0.09106
4	25	0	299	13.9865	0.09052
5	DHT	0	135	13.9963	0.13501
6	ETOH	0	161	13.5938	0.10530
7	Estr	0	151	14.2914	0.11416

Average Snout to Vent Length of Frogs by Treatment 1211

Plot of MEAN*treat. Symbol used is '*'.



Analysis of Variance for Frog Snout to Vent Length over Treatment 1212

The GLM Procedure

Class Level Information

Class	Levels	Values
treat	7	0.0 1.0 10 25 DHT ETOH Estr

Number of observations 1670

NOTE: Due to missing values, only 1661 observations can be used in this analysis.

Dependent Variable: SVL

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	6	51.322535	8.553756	3.94	0.0006
Error	1654	3590.329783	2.170695		
Corrected Total	1660	3641.652318			

R-Square	Coeff Var	Root MSE	SVL Mean
0.014093	10.56124	1.473328	13.95033

Source	DF	Type I SS	Mean Square	F Value	Pr > F
treat	6	51.32253515	8.55375586	3.94	0.0006

Source	DF	Type III SS	Mean Square	F Value	Pr > F
treat	6	51.32253515	8.55375586	3.94	0.0006

Levene's Test for Homogeneity of SVL Variance
ANOVA of Squared Deviations from Group Means

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
treat	6	81.5820	13.5970	1.10	0.3612
Error	1654	20484.3	12.3847		

Bartlett's Test for Homogeneity of SVL Variance

Source	DF	Chi-Square	Pr > ChiSq
treat	6	8.9126	0.1786

Dunnett's t Tests for SVL

NOTE: This test controls the Type I experimentwise error for comparisons of all treatments against a control.

Alpha	0.05
Error Degrees of Freedom	1654
Error Mean Square	2.170695
Critical Value of Dunnett's t	2.59779

Comparisons significant at the 0.05 level are indicated by ***.

treat Comparison	Difference Between Means	Simultaneous 95% Confidence Limits	
Estr - 0.0	0.3348	-0.0405	0.7101
10 - 0.0	0.1249	-0.1864	0.4363
DHT - 0.0	0.0397	-0.3506	0.4301
25 - 0.0	0.0299	-0.2756	0.3354
1.0 - 0.0	-0.1669	-0.4690	0.1352
ETOH - 0.0	-0.3628	-0.7308	0.0052

Nonparametric comparison of frog length over treatments 1216

The NPAR1WAY Procedure

Wilcoxon Scores (Rank Sums) for Variable SVL
Classified by Variable treat

treat	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
0.0	334	277451.50	277554.0	7761.12060	830.693114
1.0	309	243946.50	256779.0	7535.00115	789.470874
10	276	241172.50	229356.0	7207.67449	873.813406
25	296	247542.00	245976.0	7410.16568	836.290541
DHT	135	113146.50	112185.0	5291.26958	838.122222
ETOH	160	114795.50	132960.0	5713.02434	717.471875
Estr	151	142236.50	125481.0	5566.63421	941.963576

Average scores were used for ties.

Kruskal-Wallis Test

Chi-Square	22.0409
DF	6
Pr > Chi-Square	0.0012

Median Scores (Number of Points Above Median) for Variable SVL
Classified by Variable treat

treat	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
0.0	334	166.322485	166.899458	7.423923	0.497972
1.0	309	141.224852	154.406984	7.207627	0.457038
10	276	150.535503	137.916918	6.894522	0.545418
25	296	150.807692	147.910897	7.088216	0.509485
DHT	135	68.059172	67.459362	5.061379	0.504142
ETOH	160	65.059172	79.951836	5.464810	0.406620
Estr	151	87.991124	75.454545	5.324780	0.582723

Average scores were used for ties.

Median One-Way Analysis

Chi-Square	17.4213
DF	6
Pr > Chi-Square	0.0079

Percentage of Frogs Male or Female 1218

-- treat=0.0 --

The FREQ Procedure

Sex	Frequency	Percent	Cumulative Frequency	Cumulative Percent
?	2	0.60	2	0.60
F	168	50.30	170	50.90
M	164	49.10	334	100.00

Frequency Missing = 1

Percentage of Frogs Male or Female 1219

-- treat=1.0 --

The FREQ Procedure

Sex	Frequency	Percent	Cumulative Frequency	Cumulative Percent
?	3	0.97	3	0.97
F	152	49.19	155	50.16
M	154	49.84	309	100.00

Frequency Missing = 3

Percentage of Frogs Male or Female 1220

-- treat=10 --

The FREQ Procedure

Sex	Frequency	Percent	Cumulative Frequency	Cumulative Percent
?	1	0.36	1	0.36
F	142	51.45	143	51.81
M	133	48.19	276	100.00

Frequency Missing = 1

Percentage of Frogs Male or Female 1221

-- treat=25 --

The FREQ Procedure

Sex	Frequency	Percent	Cumulative Frequency	Cumulative Percent
?	14	4.73	14	4.73
F	149	50.34	163	55.07
M	133	44.93	296	100.00

Frequency Missing = 3

Percentage of Frogs Male or Female 1222

-- treat=DHT --

The FREQ Procedure

Sex	Frequency	Percent	Cumulative Frequency	Cumulative Percent
F	73	54.07	73	54.07
M	62	45.93	135	100.00

Percentage of Frogs Male or Female 1223

-- treat=ETOH --

The FREQ Procedure

Sex	Frequency	Percent	Cumulative Frequency	Cumulative Percent
F	81	50.63	81	50.63
M	79	49.38	160	100.00

Frequency Missing = 1

Percentage of Frogs Male or Female 1224

-- treat=Estr --

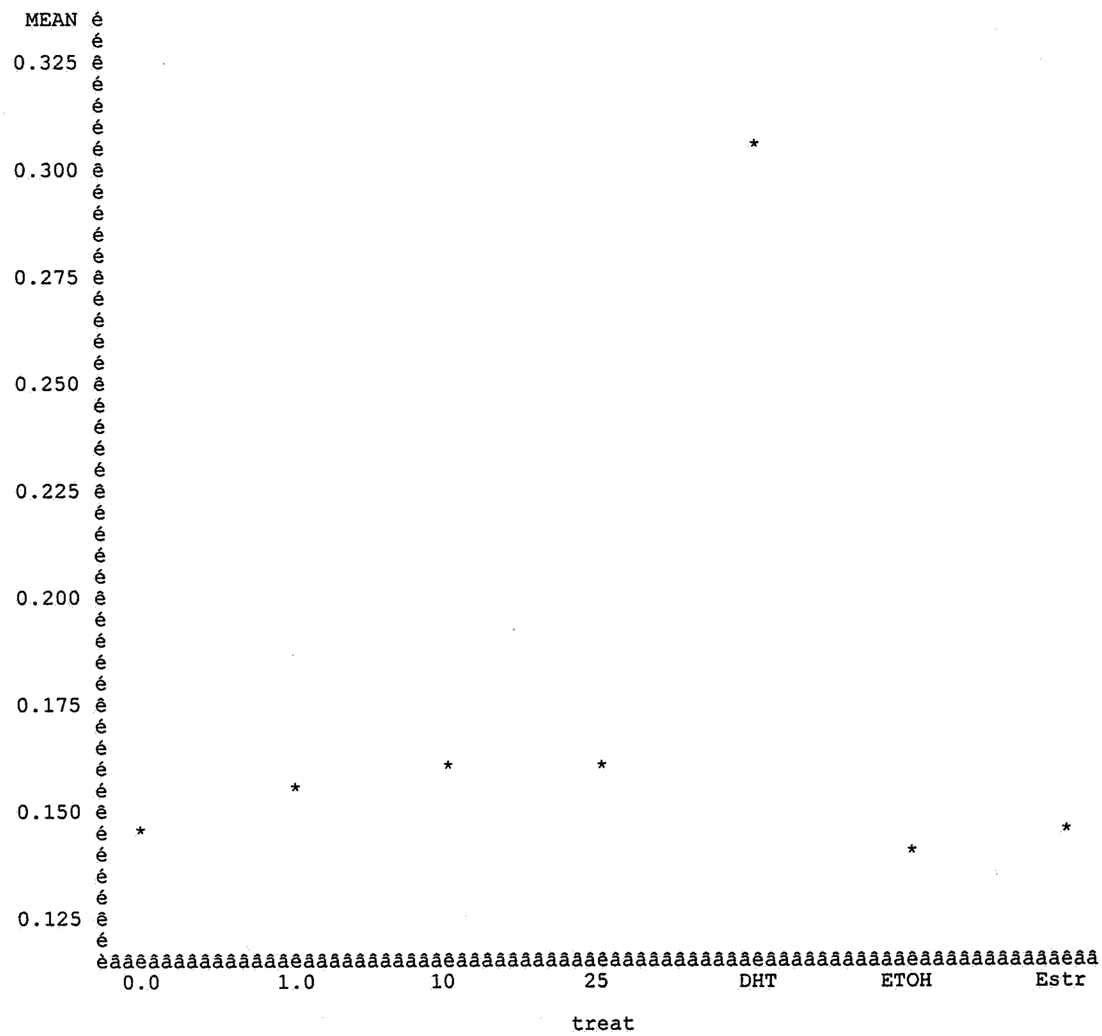
The FREQ Procedure

Sex	Frequency	Percent	Cumulative Frequency	Cumulative Percent
?	11	7.28	11	7.28
F	101	66.89	112	74.17
M	39	25.83	151	100.00

Average Total Area of Frog Larynx by Treatment 1225

Obs	treat	_TYPE_	_FREQ_	MEAN	STDERR
1	0.0	0	56	0.14694	0.003893
2	1.0	0	51	0.15720	0.005215
3	10	0	55	0.15998	0.003926
4	25	0	54	0.15752	0.004347
5	DHT	0	12	0.30669	0.012958
6	ETOH	0	12	0.14100	0.009301
7	Estr	0	12	0.14480	0.006760

Plot of MEAN*treat. Symbol used is '*'.



Analysis of Variance for Larynx Total Area over Treatment 1227

The GLM Procedure

Class Level Information

Class	Levels	Values
treat	7	0.0 1.0 10 25 DHT ETOH Estr

Number of observations 252

Dependent Variable: TOTAL

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	6	0.27515908	0.04585985	43.98	<.0001
Error	245	0.25549096	0.00104282		
Corrected Total	251	0.53065004			

R-Square	Coeff Var	Root MSE	TOTAL Mean
0.518532	20.01396	0.032293	0.161351

Source	DF	Type I SS	Mean Square	F Value	Pr > F
treat	6	0.27515908	0.04585985	43.98	<.0001

Source	DF	Type III SS	Mean Square	F Value	Pr > F
treat	6	0.27515908	0.04585985	43.98	<.0001

Levene's Test for Homogeneity of TOTAL Variance
ANOVA of Squared Deviations from Group Means

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
treat	6	0.000021	3.543E-6	1.82	0.0955
Error	245	0.000477	1.945E-6		

Bartlett's Test for Homogeneity of TOTAL Variance

Source	DF	Chi-Square	Pr > ChiSq
treat	6	9.1161	0.1672

Dunnett's t Tests for TOTAL

NOTE: This test controls the Type I experimentwise error for comparisons of all treatments against a control.

Alpha	0.05
Error Degrees of Freedom	245
Error Mean Square	0.001043
Critical Value of Dunnett's t	2.62465

Comparisons significant at the 0.05 level are indicated by ***.

treat Comparison	Difference Between Means	Simultaneous 95% Confidence Limits		
DHT - 0.0	0.159753	0.132791	0.186715	***
10 - 0.0	0.013036	-0.003054	0.029126	
25 - 0.0	0.010574	-0.005591	0.026739	
1.0 - 0.0	0.010257	-0.006148	0.026663	
Estr - 0.0	-0.002144	-0.029106	0.024817	
ETOH - 0.0	-0.005940	-0.032901	0.021022	

Nonparametric comparison of frog total larynx area over treatments 1231

The NPAR1WAY Procedure

Wilcoxon Scores (Rank Sums) for Variable TOTAL
Classified by Variable treat

treat	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
0.0	56	5953.0	7084.00	481.051626	106.303571
1.0	51	6269.0	6451.50	464.892730	122.921569
10	55	7427.0	6957.50	477.951793	135.036364
25	54	6899.0	6831.00	474.787321	127.759259
DHT	12	2956.0	1518.00	246.414285	246.333333
ETOH	12	1161.0	1518.00	246.414285	96.750000
Estr	12	1213.0	1518.00	246.414285	101.083333

Kruskal-Wallis Test

Chi-Square	41.0845
DF	6
Pr > Chi-Square	<.0001

Median Scores (Number of Points Above Median) for Variable TOTAL
Classified by Variable treat

treat	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
0.0	56	23.0	28.00	3.306398	0.410714
1.0	51	24.0	25.50	3.195334	0.470588
10	55	32.0	27.50	3.285092	0.581818
25	54	25.0	27.00	3.263342	0.462963
DHT	12	12.0	6.00	1.693672	1.000000
ETOH	12	5.0	6.00	1.693672	0.416667
Estr	12	5.0	6.00	1.693672	0.416667

Median One-Way Analysis

Chi-Square	16.3328
DF	6
Pr > Chi-Square	0.0121

Analysis of Variance for Larynx Total Area over Treatment by Sex 1233

--- Sex=F ---

The GLM Procedure

Class Level Information

Class	Levels	Values
treat	7	0.0 1.0 10 25 DHT ETOH Estr

Number of observations 62

Dependent Variable: TOTAL

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	6	0.19693080	0.03282180	43.04	<.0001
Error	55	0.04194645	0.00076266		
Corrected Total	61	0.23887726			

R-Square	Coeff Var	Root MSE	TOTAL Mean
0.824402	19.46572	0.027616	0.141872

Source	DF	Type I SS	Mean Square	F Value	Pr > F
treat	6	0.19693080	0.03282180	43.04	<.0001

Source	DF	Type III SS	Mean Square	F Value	Pr > F
treat	6	0.19693080	0.03282180	43.04	<.0001

Levene's Test for Homogeneity of TOTAL Variance
ANOVA of Squared Deviations from Group Means

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
treat	6	0.000034	5.612E-6	5.62	0.0001
Error	55	0.000055	9.985E-7		

Bartlett's Test for Homogeneity of TOTAL Variance

Source	DF	Chi-Square	Pr > ChiSq
treat	6	16.3229	0.0121

Dunnett's t Tests for TOTAL

NOTE: This test controls the Type I experimentwise error for comparisons of all treatments against a control.

Alpha	0.05
Error Degrees of Freedom	55
Error Mean Square	0.000763
Critical Value of Dunnett's t	2.67093

Comparisons significant at the 0.05 level are indicated by ***.

treat Comparison	Difference Between Means	Simultaneous 95% Confidence Limits		
DHT - 0.0	0.19547	0.15804	0.23291	***
Estr - 0.0	0.02421	-0.01322	0.06165	
10 - 0.0	0.01164	-0.01981	0.04310	
25 - 0.0	0.00455	-0.02624	0.03534	
ETOH - 0.0	0.00380	-0.03364	0.04123	
1.0 - 0.0	0.00047	-0.03176	0.03269	

-- Sex=M --

The GLM Procedure

Class Level Information

Class	Levels	Values
treat	7	0.0 1.0 10 25 DHT ETOH Estr

Number of observations 190

Dependent Variable: TOTAL

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	6	0.11691360	0.01948560	24.82	<.0001
Error	183	0.14365715	0.00078501		
Corrected Total	189	0.26057076			

R-Square	Coeff Var	Root MSE	TOTAL Mean
0.448683	16.70652	0.028018	0.167707

Source	DF	Type I SS	Mean Square	F Value	Pr > F
treat	6	0.11691360	0.01948560	24.82	<.0001

Source	DF	Type III SS	Mean Square	F Value	Pr > F
treat	6	0.11691360	0.01948560	24.82	<.0001

Levene's Test for Homogeneity of TOTAL Variance
ANOVA of Squared Deviations from Group Means

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
treat	6	9.284E-6	1.547E-6	1.20	0.3067
Error	183	0.000235	1.286E-6		

Bartlett's Test for Homogeneity of TOTAL Variance

Source	DF	Chi-Square	Pr > ChiSq
treat	6	6.4494	0.3748

Dunnett's t Tests for TOTAL

NOTE: This test controls the Type I experimentwise error for comparisons of all treatments against a control.

Alpha	0.05
Error Degrees of Freedom	183
Error Mean Square	0.000785
Critical Value of Dunnett's t	2.63648

Comparisons significant at the 0.05 level are indicated by ***.

treat Comparison	Difference Between Means	Simultaneous 95% Confidence Limits		
DHT - 0.0	0.146489	0.114385	0.178594	***
10 - 0.0	0.013549	-0.002112	0.029210	
25 - 0.0	0.013522	-0.002327	0.029371	
1.0 - 0.0	0.012629	-0.003319	0.028578	
ETOH - 0.0	0.006778	-0.025327	0.038882	
Estr - 0.0	-0.006049	-0.038154	0.026055	

Data Evaluation Report on Methods Development for the Study of Mechanism of Action of Atrazine in Adult and Metamorphosing *Xenopus laevis* and Rana clamitans: Aromatase Induction

EPA MRID Number 458677-08

Data Requirement:

EPA DP Barcode D288775

EPA MRID 458677-08
EPA Guideline 70-1(Special Study)

Test material: **Purity:** not reported

Common name Atrazine
Chemical name: IUPAC
CAS name 6-chloro-N-ethyl-N'-(1-methylethyl)-1,3,5-triazine-2,4-diamine
CAS No. 1912-24-9
Synonyms
EPA PC Code: 80803

Thomas M Steeger

Primary Reviewer: Thomas M. Steeger, Ph.D., Senior Biologist **Date:** March 31, 2003
Environmental Fate and Effects Division, ERB 4,
U. S. Environmental Protection Agency

Joseph E. Tietge

Secondary Reviewer(s): Joseph E. Tietge, M.S., Research Aquatic Biologist **Date:** 4/16/03
Mid-Continent Ecology Division, National Health and Environmental Effects Research Laboratory (Duluth),
U. S. Environmental Protection Agency

Stephanie R. Irene 5/6/03
Stephanie Irene, Ph.D., Senior Advisor **Date:**
Environmental Fate and Effects Division, ERB 3, U. S. Environmental Protection Agency

Mary Frankenberry
Mary J. Frankenberry, Senior Statistician **Date:** 5/05/03
Environmental Fate and Effects Division, ERB 3, U. S. Environmental Protection Agency

EPA PC Code 080803

Date Evaluation Completed: 05/31/2003

CITATION: Villeneuve, D. L., K. Coady, M. Hecker, M. B. Murphy, P. D. Jones and J. P. Giesy. 2003. Methods development for the study of mechanism of action of atrazine in adult and metamorphosing *Xenopus laevis* and *Rana clamitans*: aromatase induction. Aquatic Toxicology Laboratory, Michigan State University, National Food Safety and Toxicology Center, E. Lansing, MI. Sponsor: Syngenta Crop Protection, Inc., Laboratory Study ID ECORISK Number MSU-01.

EXECUTIVE SUMMARY:

In three separate studies, two involving adult male African clawed frogs (*Xenopus laevis*) and one with adult female *Xenopus*, frogs were exposed to either atrazine at 25 µg/L or to freshwater under static renewal conditions with 50% exposure solution changes every 72 hours. In the first study, 15 males were exposed for 26 days (single tank per treatment); in the second study 15 males were exposed for 43 days (single tank per treatment); and in the third study 13 females (6 in one replicate and 7 in the second replicate) were exposed for 47 days. Overall mortality was 3%, 7% and 19% in the 26, 43 and 47-day exposures, respectively; mortality was primarily associated with disease (fungal/bacterial) and was positively correlated (r=0.77) with the number of exposure days. Homogenates from a single testes and from brain were used to measure aromatase activity (CYP19 gene expression) in males. Aromatase activity in the testes was at or near the level of detection (LOD = 0.025 fmol/h/mg protein). Mean brain aromatase activity from atrazine-treated males (8.4 ± 4.2 fmol/h/mg protein) was not statistically different (p = 0.678) from controls (7.1 ± 4.2 fmol/h/mg protein) after 26 days of exposure. Following 43 days of exposure, mean brain aromatase activity in atrazine-treated males (5.8 ± 3.4 fmol/h/mg protein) was not statistically different (p = 0.199) from controls (10.4 ± 7.1 fmol/h/mg protein); however, in the second study, atrazine contaminated the controls at levels as high 0.25 µg/L. In the third exposure using female frogs, ovarian aromatase activity in atrazine-treated females averaged 4.5 ± 1.7 fmol/h/mg protein and did not differ statistically (p=0.447) from controls (5.4 ± 2.1 fmol/h/mg protein). Mean aromatase activity of brain homogenates from atrazine-treated females was 7.3 ± 5.0 fmol/h/mg protein did not differ significantly (p= 0.582) from control females (8.9 ± 4.2 fmol/h/mg protein).

With a single tank per treatment in the first two studies, it is not possible to document tank effects. Hhowever, the authors note that there was considerable variability in aromatase activity between frogs receiving the same treatment with coefficients of variability at or exceeding 100%. The third exposure where treatments were replicated confirmed that tank effects were high enough to potentially confound the ability of the study design to detect treatment effects. Additionally, with only one atrazine concentration tested, the ability of the study to discriminate potential "low-dose" effects is limited. Atrazine contamination of controls in the second study further confounded the test's ability to discriminate subtle effects.

The study recommends that future testing use replication, higher sample sizes and a broader range of atrazine levels to test for potentially subtle effects. Although not discussed in the study, water quality may have also compromised the study given the correlation of mortality with the number of days the frogs were confined and the references to the diseased state of the animals that had succumbed. Apparently, *Xenopus* are susceptible to bacterial septicemia (red-leg disease) if poor water quality persists.

Although it is indicated in the introduction that plasma sex steroids were also analyzed, these data and associated methods were not presented in this report. The only mention of sex steroid measurements was an indication that blood samples were collected by cardiac puncture.

I. MATERIALS AND METHODS

GUIDELINE FOLLOWED: Nonguideline Study
COMPLIANCE: Not conducted under full GLP; however, most practices as defined by 40 CFR Part 160, August 19, 1989 were established for this study, including but not limited to:

- Written, authorized protocol
- Written, authorized Standard Operating Procedures for all key procedures.
- Organization and Personnel were sufficient in terms of number, education, training and experience.
- Facilities were of suitable size and construction
- Equipment used was of appropriate design and adequate capacity.
- Independent A Inspection was conducted of raw data.
- Final Report was written
- Raw data, documentation, records, protocols, and final report was archieved.

A. MATERIALS:

1. Test Material Atrazine

Description: Not reported

Lot No./Batch No. : Not reported

Purity: Not reported
Stability of compound under test conditions: Not reported

Storage conditions of test chemicals:_ Not reported

2. Test organism:

Species: African clawed frog (*Xenopus laevis*)

Age at test initiation Adults
Weight at study initiation: (mean and range) Not reported
Length at study initiation: (mean and range) Not reported

Source: Adult *X. laevis* obtained from Xenopus Express® (Plant City, FL)

B. STUDY DESIGN:

Objective:

1. To test hypothesis that waterborne exposure to 25 µg/L atrazine could up-regulate aromatase activity in sexually mature *X. laevis*; male and female *X. laevis* were tested.
2. Test hypothesis that exposure to atrazine causes decreases in plasma testosterone and increases in estradiol (consistent with an increase in aromatase activity).

1. Experimental Conditions

a) Range-finding Study:

b) Definitive Study

Table 1 . Experimental Parameters

Parameter	Details
Acclimation: period: Conditions: (same as test or not) Feeding: Health: (any mortality observed)	 several weeks freshwater (treated well water) not reported mortality observed during study; frog with leision
Duration of the test	Exposure 1: 26-day Exposure 2: 43-day Exposure 3: 47-day
Test condition static/flow- through Type of dilution system for flow-through method. Renewal rate for static renewal	 static renewal NA 50% test solution change every 72 hours
Aeration, if any	not reported
Test vessel Material: (glass/stainless steel) Size: Fill volume:	 fiberglass 600 L 120 L

Parameter	Details
Source of dilution water Quality:	Treated well water (MSU-University Research Containment Facility;
<u>Water parameters</u>: Hardness pH Dissolved oxygen Total Organic carbon Particulate Matter Ammonia Nitrite Metals Pesticides Chlorine Temperature {Salinity for marine or estuarine species} Intervals of water quality measurement	no water quality data provided at all No mention of whether water quality measurements were taken at all
Number of replicates/groups: negative control: water treated ones: atrazine at 25 µg/L	Exposure 1: single tank for each treatment (25 µg atrazine/L plus control) Exposure 2: single tank for each treatment (25 µg atrazine/L plus control) Exposure 3: 2 replicates per treatment
Number of organisms per replicate /groups:	Exposure 1: 15 adult frogs/treatment Exposure 2: 15 adult frogs/treatment Exposure 3: 6 adults in Rep 1 and 7 adults in Rep 2
Biomass loading rate	Exposure 1 and 2: 15 adults/120 L = 0.13 frogs/L Exposure 3: 6 to 7 adults/120 L = 0.05 - 0.06 frogs/L
Test concentrations: nominal: measured:	25 µg/L Exposure 1: 30.6 ± 2.1 µg/L Exposure 2: 34 ± 8 µg/L Exposure 3: 32 ± 16 µg/L

Parameter	Details
Solvent (type, percentage, if used)	none
Lighting	not reported
Feeding	not reported
Recovery of chemical Level of Quantitation Level of Detection	ELISA (Envirogard Triazine®; Strategic Diagnostics Newark, DE) LOD 0.025 µg/L (Envirogard); 0.05 µg/L (Beacon)
Positive control {if used, indicate the chemical and concentrations}	none
Other parameters, if any	NA

2. Observations:

Table 2: Observations

Criteria	Details
Parameters measured including the sublethal effects/toxicity symptoms	Catalytic activity of aromatase (CYP19) measured in gonad and brain homogenates. Blood analyzed for steroid hormone levels
Observation intervals	daily
Were raw data included?	Yes
Other observations, if any	

II. RESULTS and DISCUSSION: [All results discussed in this section and the next are those reported by the study authors. Although supplemental data are typically used in a qualitative manner only, EFED verified spreadsheet data and ran basic statistical analyses on the major study parameters. See attached appendix. If results appeared to differ in any substantive way, the difference was reported in the text below.]

Although blood was collected for steroid hormone analysis, these data were not reported in this study. Catalytic activity of aromatase (CYP19) was measured using the tritiated water release assay based on method of Lephart and Simpson (1991); protein concentration was determined using fluorescamine assay based on method of Kennedy and Jones (1994). Aromatase level was expressed as fmol/h/mg protein.

Atrazine concentrations in exposure tanks are based on samples collected following solution renewal after 72-hrs static exposure Only 50% of the exposure solutions were changed at renewal though.

Exposure 1 (26 days): one atrazine-treated frog died apparently from disease (open lesions on its legs). Single testes were taken for analysis; none of the testes homogenates had measurable aromatase activity; mean aromatase activity to the testicular homogenates was not statistically different among treatments (p=0.586). Positive control (*Xenopus* ovarian tissue).

Immature *Xenopus* ovarian exhibited roughly similar aromatase levels as 2 mg/mL bovine serum albumin; whereas *X. laevis* adult ovary contained roughly 26 fmol/h/mg protein. Mean aromatase activity from atrazine-treated males (8.4 ± 4.2 fmol/h/mg protein) was not statistically different (p = 0.678) from controls (7.1 ± 4.2 fmol/h/mg protein).

Atrazine concentrations averaged 30.6 ± 2.1 µg/L, while the control tank contained less than 0.025 µg/L (LOD for ELISA).

Exposure 2 (43 days): one animal from each treatment died of unknown causes. Animals were reported to appear healthy. Testicular homogenates had low to non-detectable levels of aromatase and again, there was no statistical difference (p = 0.764) between treatments. Positive control *X. laevis* ovarian homogenates yielded measurable aromatase activity, and activity was inhibited by addition of 7.5 µL of 559 µM 4-androstein-4-ol-3, 17-dione (a specific inhibitor of aromatase).

Atrzine-treated mean brain aromatase activity (5.8 ± 3.4 fmol/h/mg protein) was not statistically different (p = 0.199) from controls (10.4 ± 7.1 fmol/h/mg protein). A negative control (*Rana pipiens* ovarian homogenate) did not yield a significant response in the aromatase assay. There were significant differences among mean aromatase activity in replicate tanks (tank effects) within each treatment group.

Atrazine concentrations over the 43-day exposure period averaged 34 ± 8 µg/L but ranged as high as 50 µg/L. Atrazine in control tanks ranged up to 0.26 µg/L but average 0.07 ± 0.09 µg/L.

Exposure 3 (47 days): two atrazine-treated and three control females died; overall mortality was 19%. Fungal or bacterial infections were considered the probable cause of the mortalities although the surviving animals are reported as appearing healthy.

Ovarian aromatase activity in atrazine-treated females averaged 4.5 ± 1.7 fmol/h/mg protein and did not differ statistically (p=0.447) from controls (5.4 ± 2.1 fmol/h/mg protein). The report notes that overall ovarian aromatase activity was approximately 33 times greater than that observed for testicular homogenates from Exposure II.

The mean aromatase activity of brain homogenates from atrazine-treated females was 7.3 ± 5.0 fmol/h/mg; protein did not differ significantly (p= 0.582) from control females (8.9 ± 4.2 fmol/h/mg protein).

Atrazine concentrations in each of the atrazine groups averaged 32 ± 16 µg/L and 28 ± 15 µg/L; however concentrations ranged as high as 70 µg/L. Atrazine concentrations in control tanks were generally less than the limit of detection and averaged 0.018 ± 0.02 µg/L and 0.016 ± 0.019 µg/L in the two replicates.

The report concludes that there were no statistically significant differences in mean brain homogenate aromatase activity among treatment groups in all three exposures (p= 0.75). For ovarian homogenates from exposure 3, there were no statistically significant differences between atrazine and control females. As a whole "the results of the three exposures do no support the hypothesis that waterborne exposure to approximately 25 µg/L atrazine causes changes in brain or gonadal aromatase activity in adult *X. laevis* relative to that in *X. laevis* exposed to laboratory water."

C. REPORTED STATISTICS:

D. VERIFICATION OF STATISTICAL RESULTS: Basic analyses in attached SAS© [Statistical Analysis System, Release 8.01, Cary, North Carolina] output)

E. STUDY DEFICIENCIES: Water quality data were not provided; the purity of atrazine was not provided; and atrazine was detected in the negative control in Exposure 2. In Exposures 1 and 3, mortality was attributed to disease. The feeding rate and lighting cycle were not discussed in the study.

F. REVIEWER'S COMMENTS:

It is unclear why a negative control (*Rana pipiens* ovarian homogenate) did not yield a significant response in the aromatase assay. In other words, why didn't aromatase from a different species convert testosterone to estrogen since the substrates are presumably the same?

In Exposure 2, atrazine in control tanks ranged up to 0.26 µg/L, but averaged 0.07 ± 0.09 µg/L. While the report states that this suggests "atrazine concentrations in control water was, on average, at least 485 times lower than that in atrazine-treated water," the levels detected were well-above the detection limit of the ELISA assay (LOD = 0.025). Atrazine in controls was at 0.1 µg/L on March 18, 0.25 µg/L on March 21, and 0.24 µg/L on March 30. Thus, exposure to atrazine in the controls was not an isolated event and may have confounded the ability of the study to detect treatment-related effects.

The level of total mortality was positively correlated (r=0.77) with the length of exposure and in most cases was associated with either bacterial and/or fungal disease.

In the second exposure, there were significant differences among mean aromatase activity in replicate tanks (tank effects) within each treatment group.

Although the report concluded that "the results of the three exposures do no support the hypothesis that waterborne exposure to approximately 25 µg/L atrazine causes changes in brain or gonadal aromatase activity in adult *X. laevis* relative to that in *X. laevis* exposed to laboratory water," the authors acknowledged that the magnitude of variability in gonadal and brain aromatase activity among individual frogs from the same treatment limited the power of the experiments to detect subtle differences even if they occurred. Large sample sizes would be required to test for subtle effects. Additionally, with only one tank per treatment in Exposures 1 and 2, the ability to account for tank effects is very limited and as the authors noted could potentially yield a false-negative or false positive response.

Furthermore, the studies only examined a single atrazine exposure concentration, i.e., 25 µg/L. The authors note that one hypothesis that has been raised is that high concentrations of atrazine may up-regulate aromatase to the point where the estrogen produced causes negative feedback on the hypothalmic-pituitary-axis resulting in down-regulation of aromatase expression. They conclude that future testing should use a wider range of concentrations.

Although it is indicated in the introduction that plasma sex steroids were also analyzed, these data and associated methods were not presented in this report. The only mention of sex steroid measurements was an indication that blood samples were collected by cardiac puncture.

G. CONCLUSIONS:

Aromatase activity in testes was too low to reliably quantify. While atrazine treatment of adult male *X. laevis* did not significantly affect brain aromatase activity relative to controls, the ability of the current studies to detect treatment effects appears limited. Since mortality over the three studies was positively correlated with the length of the study and because disease appeared to be an issue in two out of three studies, water quality may have been a problem; however, water quality data were not reported for the studies. Additionally, the second study (Exposure 2) was compromised by the discovery of atrazine in the control exposure. With a single tank per treatment (no replicates) and a single atrazine treatment (25 µg/L) per study, variability within treatments made it difficult to clearly differentiate treatment effects and to test the hypothesis.

H. REFERENCES:

Kennedy, S. W. and S. P. Jones. 1994. Simultaneous measurement of cytochrome P4501A catalyitc activity and total protein-concentration with fluorescence plate reader. Analytical Biochemistry 222(1): 217 - 223.

Laphart, E. D. and E. R. Simpson. 1991. Assay of aromatase activity. Methods in Enzymology 206: 477 - 483.

NONPARAMETRIC COMPARISON OF GONADAL AROMATASE ACTIVITY BETWEEN CONTROL AND ATRAZINE-TREATED 264

Obs	SAMPLE	_TYPE_	_FREQ_	MEAN	STD	CV
1	A1	0	2	11.1919	5.11863	45.735
2	A10	0	2	2.1999	0.02387	1.085
3	A11	0	2	1.8184	0.09016	4.958
4	A12	0	2	0.6189	0.14642	23.658
5	A13	0	2	18.8785	0.89090	4.719
6	A14	0	2	-0.3838	0.34889	-90.914
7	A15	0	2	-1.7780	0.36618	-20.595
8	A2	0	2	19.5097	0.08537	0.438
9	A3	0	2	1.0145	0.35979	35.465
10	A4	0	2	13.9604	0.21978	1.574
11	A7	0	2	17.5941	0.01244	0.071
12	A8	0	2	6.4782	1.36797	21.116
13	A9	0	2	17.6115	0.57149	3.245
14	C1	0	2	-0.8158	0.25213	-30.907
15	C10	0	2	12.0858	0.74018	6.124
16	C11	0	2	14.7327	0.07916	0.537
17	C12	0	2	3.1996	0.31515	9.850
18	C13	0	2	7.2257	0.47387	6.558
19	C14	0	2	0.5245	0.04568	8.709
20	C15	0	2	12.5170	0.11766	0.940
21	C2	0	2	-0.0897	0.25792	-287.405
22	C3	0	2	14.6997	5.50738	37.466
23	C4	0	2	1.7527	0.36438	20.790
24	C5	0	2	1.2051	1.46082	121.218
25	C6	0	2	13.9926	3.28426	23.471
26	C8	0	2	18.9569	0.13915	0.734
27	C9	0	2	-0.2912	0.07412	-25.456

MEAN BRAIN AROMATASE ACTIVITY IN CONTROL AND ATRAZINE-TREATED FROGS (EXP 1) 265

Obs	GROUP	_TYPE_	_FREQ_	MEAN	STD	CV
1	ATRAZIN	0	13	8.36265	8.27945	99.0051
2	CONTROL	0	14	7.12111	7.06613	99.2279

ANOVA FOR BRAIN AROMATASE ACTIVITY BETWEEN CONTROL AND ATRAZINE-TREATED FROGS (EXP 1) 266

The ANOVA Procedure

Class Level Information

Class	Levels	Values
GROUP	2	ATRAZIN CONTROL

Number of observations 27

Dependent Variable: MEAN

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	1	10.390314	10.390314	0.18	0.6780
Error	25	1471.684807	58.867392		
Corrected Total	26	1482.075121			

R-Square	Coeff Var	Root MSE	MEAN Mean
0.007011	99.39913	7.672509	7.718889

Source	DF	Anova SS	Mean Square	F Value	Pr > F
GROUP	1	10.39031393	10.39031393	0.18	0.6780

Levene's Test for Homogeneity of MEAN Variance
ANOVA of Squared Deviations from Group Means

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
GROUP	1	1928.1	1928.1	1.54	0.2255
Error	25	31216.8	1248.7		

Bartlett's Test for Homogeneity of MEAN Variance

Source	DF	Chi-Square	Pr > ChiSq
GROUP	1	0.3013	0.5831

Dunnett's t Tests for MEAN

NOTE: This test controls the Type I experimentwise error for comparisons of all treatments against a control.

Alpha	0.05
Error Degrees of Freedom	25
Error Mean Square	58.86739
Critical Value of Dunnett's t	2.05959

Comparisons significant at the 0.05 level are indicated by ***.

GROUP Comparison	Difference Between Means	Simultaneous 95% Confidence Limits	
CONTROL - ATRAZIN	-1.242	-7.328	4.845

NONPARAMETRIC COMPARISON OF BRAIN AROMATASE ACTIVITY BETWEEN CONTROL AND ATRAZINE-TREATED FR 270

The NPAR1WAY Procedure

Wilcoxon Scores (Rank Sums) for Variable MEAN
Classified by Variable GROUP

GROUP	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
ATRAZIN	13	190.0	182.0	20.607442	14.615385
CONTROL	14	188.0	196.0	20.607442	13.428571

Wilcoxon Two-Sample Test

Statistic	190.0000
Normal Approximation	
Z	0.3639
One-Sided Pr > Z	0.3579
Two-Sided Pr > |Z|	0.7159
t Approximation	
One-Sided Pr > Z	0.3594
Two-Sided Pr > |Z|	0.7188

Z includes a continuity correction of 0.5.

Kruskal-Wallis Test

Chi-Square	0.1507
DF	1
Pr > Chi-Square	0.6979

Median Scores (Number of Points Above Median) for Variable MEAN
Classified by Variable GROUP

GROUP	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
ATRAZIN	13	6.0	6.259259	1.321968	0.461538
CONTROL	14	7.0	6.740741	1.321968	0.500000

Median Two-Sample Test

Statistic	6.0000
Z	-0.1961
One-Sided Pr < Z	0.4223
Two-Sided Pr > |Z|	0.8445

Median One-Way Analysis

Chi-Square	0.0385
DF	1
Pr > Chi-Square	0.8445

MEAN GONADAL AROMATASE ACTIVITY IN CONTROL AND ATRAZINE-TREATED FROGS (EXP 1) 273

Obs	SAMPLE	_TYPE_	_FREQ_	MEAN	STD	CV
1	A1	0	2	0.40032	1.40426	350.79
2	A10	0	2	-0.06537	0.04967	-75.99
3	A11	0	2	0.00952	0.22021	2314.17
4	A12	0	2	-0.33007	0.17387	-52.68
5	A13	0	2	-0.04765	0.14865	-311.96
6	A14	0	2	-0.41950	0.00438	-1.04
7	A15	0	2	-0.14911	0.14151	-94.90
8	A2	0	2	-0.52470	0.90598	-172.67
9	A3	0	2	-0.39147	0.30402	-77.66
10	A4	0	2	-0.47445	0.00000	0.00
11	A5	0	2	0.11161	0.20003	179.21
12	A7	0	2	-0.21175	0.02073	-9.79
13	A8	0	2	-0.34509	0.02312	-6.70
14	A9	0	2	-0.08582	0.10868	-126.65
15	C1	0	2	-0.59029	0.29282	-49.61
16	C10	0	2	-0.84140	0.11866	-14.10
17	C11	0	2	-0.01878	0.11230	-597.83
18	C12	0	2	-0.19693	0.05968	-30.30
19	C13	0	2	-0.05506	0.02920	-53.03
20	C14	0	2	-0.03542	0.36070	-1018.23
21	C15	0	2	0.40039	0.08395	20.97
22	C2	0	2	-0.46908	0.05875	-12.52
23	C3	0	2	-0.27553	0.13632	-49.48
24	C4	0	2	-0.38774	0.48604	-125.35
25	C5	0	2	-0.03013	0.00000	0.00
26	C6	0	2	-0.27841	0.00000	0.00
27	C8	0	2	-0.33768	0.12295	-36.41
28	C9	0	2	-0.21742	0.07923	-36.44

MEAN GONADAL AROMATASE ACTIVITY IN CONTROL AND ATRAZINE-TREATED FROGS (EXP 1) 273

Obs	GROUP	_TYPE_	_FREQ_	MEAN	STD	CV
1	ATRAZIN	0	14	-0.18025	0.25669	-142.408
2	CONTROL	0	14	-0.23811	0.29774	-125.044

ANOVA FOR GONADAL AROMATASE ACTIVITY BETWEEN CONTROL AND ATRAZINE-TREATED FROGS (EXP 1) 274

The ANOVA Procedure

Class Level Information

Class	Levels	Values
GROUP	2	ATRAZIN CONTROL

Number of observations 28

Dependent Variable: MEAN

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	1	0.02342954	0.02342954	0.30	0.5866
Error	26	2.00900245	0.07726932		
Corrected Total	27	2.03243199			

R-Square	Coeff Var	Root MSE	MEAN Mean
0.011528	-132.8879	0.277974	-0.209179

Source	DF	Anova SS	Mean Square	F Value	Pr > F
GROUP	1	0.02342954	0.02342954	0.30	0.5866

Levene's Test for Homogeneity of MEAN Variance
ANOVA of Squared Deviations from Group Means

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
GROUP	1	0.00313	0.00313	0.25	0.6229
Error	26	0.3280	0.0126		

Bartlett's Test for Homogeneity of MEAN Variance

Source	DF	Chi-Square	Pr > ChiSq
GROUP	1	0.2744	0.6004

Dunnett's t Tests for MEAN

NOTE: This test controls the Type I experimentwise error for comparisons of all treatments against a control.

Alpha	0.05
Error Degrees of Freedom	26
Error Mean Square	0.077269
Critical Value of Dunnett's t	2.05558
Minimum Significant Difference	0.216

Comparisons significant at the 0.05 level are indicated by ***.

GROUP Comparison	Difference Between Means	Simultaneous 95% Confidence Limits	
CONTROL - ATRAZIN	-0.05785	-0.27382	0.15811

NONPARAMETRIC COMPARISON OF GONADAL AROMATASE ACTIVITY BETWEEN CONTROL AND ATRAZINE-TREATED 278

The NPAR1WAY Procedure

Wilcoxon Scores (Rank Sums) for Variable MEAN
Classified by Variable GROUP

GROUP	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
ATRAZIN	14	208.0	203.0	21.763884	14.857143
CONTROL	14	198.0	203.0	21.763884	14.142857

Wilcoxon Two-Sample Test

Statistic	208.0000
Normal Approximation	
Z	0.2068
One-Sided Pr > Z	0.4181
Two-Sided Pr > |Z|	0.8362
t Approximation	
One-Sided Pr > Z	0.4189
Two-Sided Pr > |Z|	0.8377

Z includes a continuity correction of 0.5.

Kruskal-Wallis Test

Chi-Square	0.0528
DF	1
Pr > Chi-Square	0.8183

Median Scores (Number of Points Above Median) for Variable MEAN
Classified by Variable GROUP

GROUP	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
ATRAZIN	14	8.0	7.0	1.347151	0.571429
CONTROL	14	6.0	7.0	1.347151	0.428571

Median Two-Sample Test

Statistic	8.0000
Z	0.7423
One-Sided Pr > Z	0.2290
Two-Sided Pr > |Z|	0.4579

Median One-Way Analysis

Chi-Square	0.5510
DF	1
Pr > Chi-Square	0.4579

AVERAGE ATRAZINE CONCENTRATIONS IN CONTROL EXPOSURE TANKS BY DATE 280

Obs	MONTH	DAY	_TYPE_	_FREQ_	MEAN
1	3	3	0	2	0.03820
2	3	6	0	2	0.06553
3	3	9	0	2	0.01873
4	3	12	0	2	0.00515
5	3	15	0	2	0.03088
6	3	18	0	2	0.11453
7	3	21	0	2	0.26317
8	3	24	0	2	0.01652
9	3	30	0	2	0.22902
10	4	5	0	2	0.00473
11	4	8	0	2	0.00725

AVERAGE ATRAZINE CONCENTRATION IN CONTROL EXPOSURE TANK ACROSS ENTIRE STUDY (EXP 1) 281

Obs	_TYPE_	_FREQ_	MEAN	STD	CV
1	0	11	0.072156	0.092187	127.761

AVERAGE ATRAZINE CONCENTRATIONS IN 25 UG/L EXPOSURE 1 TANKS BY DATE 282

Obs	MONTH	DAY	_TYPE_	_FREQ_	MEAN
1	3	3	0	2	22.5051
2	3	6	0	2	32.4627
3	3	9	0	2	25.4194
4	3	12	0	2	25.8225
5	3	15	0	2	38.2852
6	3	18	0	2	34.8589
7	3	21	0	2	44.3471
8	3	24	0	2	49.5251
9	3	30	0	2	35.0879
10	4	5	0	2	33.9842
11	4	8	0	2	32.9775

AVERAGE ATRAZINE CONCENTRATION IN 25 UG/L EXPOSURE TANK ACROSS ENTIRE STUDY 283

Obs	_TYPE_	_FREQ_	MEAN	STD	CV
1	0	11	34.1160	8.02663	23.5275

MEAN BRAIN AROMATASE ACTIVITY FOLLOWING EXPOSURE 2 284

Obs	_TYPE_	_FREQ_	MEAN	STD	CV
1	0	28	8.07322	9.41514	116.622

ANOVA FOR BRAIN AROMATASE ACTIVITY BETWEEN CONTROL AND ATRAZINE-TREATED FROGS (EXPOSURE 2 285

The ANOVA Procedure

Class Level Information

Class	Levels	Values
GROUP	2	EXP REF

Number of observations 28

Dependent Variable: FMOL

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	1	150.032040	150.032040	1.74	0.1988
Error	26	2243.380177	86.283853		
Corrected Total	27	2393.412216			

R-Square	Coeff Var	Root MSE	FMOL Mean
0.062685	115.0583	9.288910	8.073222

Source	DF	Anova SS	Mean Square	F Value	Pr > F
GROUP	1	150.0320396	150.0320396	1.74	0.1988

Levene's Test for Homogeneity of FMOL Variance
ANOVA of Squared Deviations from Group Means

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
GROUP	1	70229.1	70229.1	3.77	0.0632
Error	26	484906	18650.2		

Bartlett's Test for Homogeneity of FMOL Variance

Source	DF	Chi-Square	Pr > ChiSq
GROUP	1	6.2027	0.0128

Dunnett's t Tests for FMOL

NOTE: This test controls the Type I experimentwise error for comparisons of all treatments against a control.

Alpha	0.05
Error Degrees of Freedom	26
Error Mean Square	86.28385
Critical Value of Dunnett's t	2.05558
Minimum Significant Difference	7.2169

Comparisons significant at the 0.05 level are indicated by ***.

GROUP Comparison	Difference Between Means	Simultaneous 95% Confidence Limits	
REF - EXP	4.630	-2.587	11.846

NONPARAMETRIC COMPARISON OF BRAIN AROMATASE ACTIVITY BETWEEN CONTROL AND ATRAZINE-TREATED FR 289

The NPAR1WAY Procedure

Wilcoxon Scores (Rank Sums) for Variable FMOL
Classified by Variable GROUP

GROUP	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
EXP	14	186.0	203.0	21.763884	13.285714
REF	14	220.0	203.0	21.763884	15.714286

Wilcoxon Two-Sample Test

Statistic	186.0000
Normal Approximation	
Z	-0.7581
One-Sided Pr < Z	0.2242
Two-Sided Pr > |Z|	0.4484
t Approximation	
One-Sided Pr < Z	0.2275
Two-Sided Pr > |Z|	0.4549

Z includes a continuity correction of 0.5.

Kruskal-Wallis Test

Chi-Square	0.6101
DF	1
Pr > Chi-Square	0.4347

Median Scores (Number of Points Above Median) for Variable FMOL
Classified by Variable GROUP

GROUP	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
EXP	14	6.0	7.0	1.347151	0.428571
REF	14	8.0	7.0	1.347151	0.571429

Median Two-Sample Test

Statistic	6.0000
Z	-0.7423
One-Sided Pr < Z	0.2290
Two-Sided Pr > |Z|	0.4579

Median One-Way Analysis

Chi-Square	0.5510
DF	1
Pr > Chi-Square	0.4579

MEAN ADULT MALE GONADAL AROMATASE ACTIVITY IN ATRAZINE-TREATED AND CONTROL FROGS (EXP 2) 291

Obs	GROUP	_TYPE_	_FREQ_	MEAN	STD	CV
1	ATRAZIN	0	14	0.13818	0.09607	69.525
2	CONTROL	0	14	0.15601	0.19786	126.827

ANOVA FOR ADULT MALE GONADAL AROMATASE ACTIVITY BETWEEN CONTROL AND ATRAZINE-TREATED FROGS (292

The ANOVA Procedure

Class Level Information

Class	Levels	Values
GROUP	2	ATRAZIN CONTROL

Number of observations 28

Dependent Variable: FMOL

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	1	0.00222571	0.00222571	0.09	0.7640
Error	26	0.62891899	0.02418919		
Corrected Total	27	0.63114470			

R-Square	Coeff Var	Root MSE	FMOL Mean
0.003526	105.7346	0.155529	0.147094

Source	DF	Anova SS	Mean Square	F Value	Pr > F
GROUP	1	0.00222571	0.00222571	0.09	0.7640

Levene's Test for Homogeneity of FMOL Variance
ANOVA of Squared Deviations from Group Means

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
GROUP	1	0.00540	0.00540	1.08	0.3074
Error	26	0.1296	0.00499		

Bartlett's Test for Homogeneity of FMOL Variance

Source	DF	Chi-Square	Pr > ChiSq
GROUP	1	6.0347	0.0140

Dunnett's t Tests for FMOL

NOTE: This test controls the Type I experimentwise error for comparisons of all treatments against a control.

Alpha	0.05
Error Degrees of Freedom	26
Error Mean Square	0.024189
Critical Value of Dunnett's t	2.05558
Minimum Significant Difference	0.1208

Comparisons significant at the 0.05 level are indicated by ***.

GROUP Comparison	Difference Between Means	Simultaneous 95% Confidence Limits	
CONTROL - ATRAZIN	0.01783	-0.10300	0.13867

NONPARAMETRIC COMPARISON OF ADULT MALE GONADAL AROMATASE ACTIVITY BETWEEN CONTROL AND ATRAZI 296

The NPAR1WAY Procedure

Wilcoxon Scores (Rank Sums) for Variable FMOL
Classified by Variable GROUP

GROUP	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
ATRAZIN	14	224.0	203.0	21.763884	16.0
CONTROL	14	182.0	203.0	21.763884	13.0

Wilcoxon Two-Sample Test

Statistic	224.0000
Normal Approximation	
Z	0.9419
One-Sided Pr > Z	0.1731
Two-Sided Pr > |Z|	0.3462
t Approximation	
One-Sided Pr > Z	0.1773
Two-Sided Pr > |Z|	0.3546

Z includes a continuity correction of 0.5.

Kruskal-Wallis Test

Chi-Square	0.9310
DF	1
Pr > Chi-Square	0.3346

Median Scores (Number of Points Above Median) for Variable FMOL
Classified by Variable GROUP

GROUP	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
ATRAZIN	14	9.0	7.0	1.347151	0.642857
CONTROL	14	5.0	7.0	1.347151	0.357143

Median Two-Sample Test

Statistic	9.0000
Z	1.4846
One-Sided Pr > Z	0.0688
Two-Sided Pr > |Z|	0.1376

Median One-Way Analysis

Chi-Square	2.2041
DF	1
Pr > Chi-Square	0.1376

MEAN ATRAZINE LEVEL BY SAMPLING DATE FOR CONTROL (C) AND ATRAZINE (A) TREATED TANKS 298

Obs	MONTH	DAY	SAMPLE	GROUP	_TYPE_	_FREQ_	MEAN	STD	CV
1	4	17	A1	ATRAZINE	0	2	21.3668	0.25186	1.1788
2	4	17	A2	ATRAZINE	0	2	6.8501	0.20184	2.9465
3	4	17	C1	CONTROL	0	2	0.0072	0.00365	50.6899
4	4	17	C2	CONTROL	0	2	0.0041	0.00070	17.0096
5	4	20	A1	ATRAZINE	0	2	22.4385	2.50752	11.1751
6	4	20	A2	ATRAZINE	0	2	4.5276	1.69332	37.3999
7	4	20	C1	CONTROL	0	2	0.0070	0.00132	18.7495
8	4	20	C2	CONTROL	0	2	0.0254	0.00179	7.0668
9	4	23	A1	ATRAZINE	0	2	18.2464	3.42110	18.7495
10	4	23	A2	ATRAZINE	0	2	32.0647	4.89450	15.2644
11	4	23	C1	CONTROL	0	2	0.0769	0.00724	9.4163
12	4	23	C2	CONTROL	0	2	0.0771	0.00045	0.5894
13	5	2	A1	ATRAZINE	0	2	25.6397	2.86526	11.1751
14	5	2	A2	ATRAZINE	0	2	17.2724	6.55444	37.9475
15	5	2	C1	CONTROL	0	2	0.0234	0.00963	41.2067
16	5	2	C2	CONTROL	0	2	0.0186	0.00865	46.5324
17	5	5	A1	ATRAZINE	0	2	17.7872	4.56176	25.6463
18	5	5	A2	ATRAZINE	0	2	17.4941	5.47574	31.3004
19	5	5	C1	CONTROL	0	2	0.0057	0.00027	4.7134
20	5	5	C2	CONTROL	0	2	0.0044	0.00008	1.7681
21	5	8	A1	ATRAZINE	0	2	21.1917	0.49956	2.3573
22	5	8	C1	CONTROL	0	2	0.0068	0.00052	7.6546
23	5	8	C2	CONTROL	0	2	0.0048	0.00057	11.7606
24	5	11	C1	CONTROL	0	2	0.0636	0.01413	22.2114
25	5	11	C2	CONTROL	0	2	0.0121	0.00445	36.8512
26	5	14	C1	CONTROL	0	2	0.0202	0.00107	5.3020
27	5	14	C2	CONTROL	0	2	0.0140	0.00530	37.9475
28	5	17	C1	CONTROL	0	2	0.0064	0.00300	46.5324
29	5	17	C2	CONTROL	0	2	0.0072	0.00025	3.5356
30	5	20	C1	CONTROL	0	2	0.0058	0.00219	37.9475
31	5	20	C2	CONTROL	0	2	0.0040	0.00073	18.1701
32	5	23	C1	CONTROL	0	2	0.0110	0.00672	61.1231
33	5	23	C2	CONTROL	0	2	0.0203	0.00892	43.8868
34	5	26	C1	CONTROL	0	2	0.0094	0.00094	10.0029
35	5	26	C2	CONTROL	0	2	0.0177	0.00239	13.5146
36	5	29	C1	CONTROL	0	2	0.0055	0.00197	35.7501
37	5	29	C2	CONTROL	0	2	0.0055	0.00065	11.7606
38	6	1	C1	CONTROL	0	2	0.0064	0.00101	15.8467
39	6	1	C2	CONTROL	0	2	0.0047	0.00087	18.7494

MEAN ATRAZINE CONCENTRATION ACROSS SAMPLING DATES FOR EACH OF THE TWO CONTROL (C) AND ATRAZI 299

Obs	SAMPLE	GROUP	_TYPE_	_FREQ_	MEAN	STD	CV
1	A1	ATRAZINE	0	6	21.1117	2.8844	13.662
2	A2	ATRAZINE	0	5	15.6418	10.9158	69.786
3	C1	CONTROL	0	14	0.0182	0.0229	125.436
4	C2	CONTROL	0	14	0.0157	0.0191	121.373

MEAN ATRAZINE CONCENTRATION IN CONTROL AND ATRAZINE-TREATED TANKS OVER ENTIRE STUDY (EXP 3 300

Obs	GROUP	_TYPE_	_FREQ_	MEAN	MIN	MAX	STD	CV
1	ATRAZINE	0	11	18.6254	4.52761	32.0647	7.74480	41.582
2	CONTROL	0	28	0.0170	0.00403	0.0771	0.02070	121.980

MEAN BRAIN AROMATASE ACTIVITY BY SAMPLE AND NUMBER IN CONTROL (C) AND ATRAZINE-TREATETED (A) 301

Obs	SAMPLE	NUMBER	GROUP	_TYPE_	_FREQ_	MEAN	STD	CV
1	A1	1	ATRAZINE	0	2	20.9131	0.52971	2.5329
2	A1	2	ATRAZINE	0	2	1.9509	0.02533	1.2982
3	A1	3	ATRAZINE	0	2	1.3884	0.00843	0.6070
4	A1	4	ATRAZINE	0	2	3.8467	0.18493	4.8075
5	A1	5	ATRAZINE	0	2	5.6692	0.02706	0.4774
6	A1	6	ATRAZINE	0	2	0.9863	0.02995	3.0367
7	A2	1	ATRAZINE	0	2	7.5578	0.14937	1.9764
8	A2	2	ATRAZINE	0	2	8.7285	1.14801	13.1524
9	A2	3	ATRAZINE	0	2	7.6883	0.05418	0.7047
10	A2	4	ATRAZINE	0	2	-0.0514	0.00540	-10.5056
11	A2	5	ATRAZINE	0	2	21.1885	0.02985	0.1409
12	C1	1	CONTROL	0	2	11.8105	0.01860	0.1575
13	C1	2	CONTROL	0	2	2.1175	0.20755	9.8015
14	C1	3	CONTROL	0	2	6.2637	0.10634	1.6977
15	C1	4	CONTROL	0	2	21.4478	0.23323	1.0874
16	C1	5	CONTROL	0	2	4.1599	0.02262	0.5438
17	C2	1	CONTROL	0	2	15.0054	2.98537	19.8953
18	C2	2	CONTROL	0	2	1.0907	0.04346	3.9843
19	C2	3	CONTROL	0	2	7.0063	0.10293	1.4692
20	C2	4	CONTROL	0	2	8.3976	0.10458	1.2454
21	C2	5	CONTROL	0	2	12.2114	0.15241	1.2481

MEAN BRAIN AROMATASE ACTIVITY BY SAMPLE IN CONTROL AND ATRAZINE-TREATED FROGS (EXP 3) 302

Obs	SAMPLE	_TYPE_	_FREQ_	MEAN	STD	CV
1	A1	0	6	5.79244	7.61168	131.407
2	A2	0	5	9.02234	7.65441	84.838
3	C1	0	5	9.15987	7.76212	84.741
4	C2	0	5	8.74230	5.31368	60.781

MEAN BRAIN AROMATASE ACTIVITY IN CONTROL AND ATRAZINE-TREATED FROGS (EXP 3) 303

Obs	GROUP	_TYPE_	_FREQ_	MEAN	STD	CV
1	ATRAZINE	0	11	7.26057	7.43303	102.375
2	CONTROL	0	10	8.95109	6.27499	70.103

ANOVA FOR BRAIN AROMATASE ACTIVITY BETWEEN CONTROL AND ATRAZINE-TREATED FROGS (EXP 3) 304

The ANOVA Procedure

Class Level Information

Class	Levels	Values
GROUP	2	ATRAZINE CONTROL

Number of observations 21

Dependent Variable: MEAN

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	1	14.9695821	14.9695821	0.31	0.5820
Error	19	906.8789562	47.7304714		
Corrected Total	20	921.8485383			

R-Square	Coeff Var	Root MSE	MEAN Mean
0.016239	85.65689	6.908724	8.065579

Source	DF	Anova SS	Mean Square	F Value	Pr > F
GROUP	1	14.96958206	14.96958206	0.31	0.5820

Levene's Test for Homogeneity of MEAN Variance
ANOVA of Squared Deviations from Group Means

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
GROUP	1	1145.7	1145.7	0.31	0.5865
Error	19	71107.0	3742.5		

Bartlett's Test for Homogeneity of MEAN Variance

Source	DF	Chi-Square	Pr > ChiSq
GROUP	1	0.2554	0.6133

Dunnett's t Tests for MEAN

NOTE: This test controls the Type I experimentwise error for comparisons of all treatments against a control.

Alpha	0.05
Error Degrees of Freedom	19
Error Mean Square	47.73047
Critical Value of Dunnett's t	2.09309

Comparisons significant at the 0.05 level are indicated by ***.

GROUP Comparison	Difference Between Means	Simultaneous 95% Confidence Limits	
CONTROL - ATRAZINE	1.691	-4.628	8.009

NONPARAMETRIC COMPARISON OF BRAIN AROMATASE ACTIVITY BETWEEN CONTROL AND ATRAZINE-TREATED FR 308

The NPAR1WAY Procedure

Wilcoxon Scores (Rank Sums) for Variable MEAN
Classified by Variable GROUP

GROUP	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
ATRAZINE	11	107.0	121.0	14.200939	9.727273
CONTROL	10	124.0	110.0	14.200939	12.400000

Wilcoxon Two-Sample Test

Statistic	124.0000
Normal Approximation	
Z	0.9506
One-Sided Pr > Z	0.1709
Two-Sided Pr > |Z|	0.3418
t Approximation	
One-Sided Pr > Z	0.1766
Two-Sided Pr > |Z|	0.3531

Z includes a continuity correction of 0.5.

Kruskal-Wallis Test

Chi-Square	0.9719
DF	1
Pr > Chi-Square	0.3242

Median Scores (Number of Points Above Median) for Variable MEAN
Classified by Variable GROUP

GROUP	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
ATRAZINE	11	5.0	5.238095	1.171274	0.454545
CONTROL	10	5.0	4.761905	1.171274	0.500000

Median Two-Sample Test

Statistic	5.0000
Z	0.2033
One-Sided Pr > Z	0.4195
Two-Sided Pr > |Z|	0.8389

Median One-Way Analysis

Chi-Square	0.0413
DF	1
Pr > Chi-Square	0.8389

MEAN GONADAL AROMATASE ACTIVITY BY SAMPLE AND NUMBER IN CONTROL (C) AND ATRAZINE-TREATETED (310

Obs	SAMPLE	NUMBER	GROUP	_TYPE_	_FREQ_	MEAN	STD	CV
1	A1	1	ATRAZINE	0	2	4.3309	0.13618	3.1445
2	A1	2	ATRAZINE	0	2	3.8744	0.02522	0.6510
3	A1	3	ATRAZINE	0	2	7.3398	0.02316	0.3155
4	A1	4	ATRAZINE	0	2	5.1150	0.06204	1.2129
5	A1	5	ATRAZINE	0	2	8.3364	0.01106	0.1327
6	A1	6	ATRAZINE	0	2	7.3282	0.02467	0.3366
7	A2	1	ATRAZINE	0	2	1.7495	0.02643	1.5105
8	A2	2	ATRAZINE	0	2	4.9783	0.12473	2.5055
9	A2	3	ATRAZINE	0	2	3.3030	0.07831	2.3710
10	A2	4	ATRAZINE	0	2	0.2169	0.02263	10.4327
11	A2	5	ATRAZINE	0	2	2.8070	0.34990	12.4655
12	C1	1	CONTROL	0	2	3.5497	0.02948	0.8304
13	C1	2	CONTROL	0	2	1.6790	0.04290	2.5551
14	C1	3	CONTROL	0	2	4.5738	0.50058	10.9444
15	C1	4	CONTROL	0	2	3.9795	0.02188	0.5497
16	C1	5	CONTROL	0	2	3.1596	0.03938	1.2463
17	C2	1	CONTROL	0	2	10.6332	0.14104	1.3264
18	C2	2	CONTROL	0	2	9.6644	0.09341	0.9665
19	C2	3	CONTROL	0	2	3.3400	1.00685	30.1450
20	C2	4	CONTROL	0	2	6.0585	0.00074	0.0122
21	C2	5	CONTROL	0	2	7.5248	0.31761	4.2209

MEAN GONADAL AROMATASE ACTIVITY BY SAMPLE IN CONTROL AND ATRAZINE-TREATED FROGS (EXP 3) 311

Obs	SAMPLE	_TYPE_	_FREQ_	MEAN	STD	CV
1	A1	0	6	6.05412	1.84866	30.5355
2	A2	0	5	2.61093	1.77449	67.9639
3	C1	0	5	3.38835	1.09028	32.1774
4	C2	0	5	7.44418	2.90987	39.0892

MEAN GONADAL AROMATASE ACTIVITY IN CONTROL AND ATRAZINE-TREATED FROGS (EXP 3) 312

Obs	GROUP	_TYPE_	_FREQ_	MEAN	STD	CV
1	ATRAZINE	0	11	4.48904	2.49031	55.4753
2	CONTROL	0	10	5.41627	2.97674	54.9592

ANOVA FOR GONADAL AROMATASE ACTIVITY BETWEEN CONTROL AND ATRAZINE-TREATED FROGS (EXP 3) 313

The ANOVA Procedure

Class Level Information

Class	Levels	Values
GROUP	2	ATRAZINE CONTROL

Number of observations 21

Dependent Variable: MEAN

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	1	4.5034675	4.5034675	0.60	0.4468
Error	19	141.7650045	7.4613160		
Corrected Total	20	146.2684720			

R-Square	Coeff Var	Root MSE	MEAN Mean
0.030789	55.40006	2.731541	4.930574

Source	DF	Anova SS	Mean Square	F Value	Pr > F
GROUP	1	4.50346755	4.50346755	0.60	0.4468

Levene's Test for Homogeneity of MEAN Variance
ANOVA of Squared Deviations from Group Means

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
GROUP	1	28.6086	28.6086	0.49	0.4920
Error	19	1107.1	58.2672		

Bartlett's Test for Homogeneity of MEAN Variance

Source	DF	Chi-Square	Pr > ChiSq
GROUP	1	0.2867	0.5923

Dunnett's t Tests for MEAN

NOTE: This test controls the Type I experimentwise error for comparisons of all treatments against a control.

Alpha	0.05
Error Degrees of Freedom	19
Error Mean Square	7.461316
Critical Value of Dunnett's t	2.09309

Comparisons significant at the 0.05 level are indicated by ***.

GROUP Comparison	Difference Between Means	Simultaneous 95% Confidence Limits	
CONTROL - ATRAZINE	0.9272	-1.5709	3.4253

NONPARAMETRIC COMPARISON OF GONADAL AROMATASE ACTIVITY BETWEEN CONTROL AND ATRAZINE-TREATED 317

The NPAR1WAY Procedure

Wilcoxon Scores (Rank Sums) for Variable MEAN
Classified by Variable GROUP

GROUP	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
ATRAZINE	11	113.0	121.0	14.200939	10.272727
CONTROL	10	118.0	110.0	14.200939	11.800000

Wilcoxon Two-Sample Test

Statistic	118.0000
Normal Approximation	
Z	0.5281
One-Sided Pr > Z	0.2987
Two-Sided Pr > |Z|	0.5974
t Approximation	
One-Sided Pr > Z	0.3016
Two-Sided Pr > |Z|	0.6032

Z includes a continuity correction of 0.5.

Kruskal-Wallis Test

Chi-Square	0.3174
DF	1
Pr > Chi-Square	0.5732

Median Scores (Number of Points Above Median) for Variable MEAN
Classified by Variable GROUP

GROUP	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
ATRAZINE	11	5.0	5.238095	1.171274	0.454545
CONTROL	10	5.0	4.761905	1.171274	0.500000

Median Two-Sample Test

Statistic	5.0000
Z	0.2033
One-Sided Pr > Z	0.4195
Two-Sided Pr > |Z|	0.8389

Median One-Way Analysis

Chi-Square	0.0413
DF	1
Pr > Chi-Square	0.8389

Data Evaluation Report on Field Exposure of *Xenopus laevis* to Atrazine and Other Triazines in South Africa: Feasibility Study for Site Characterization and Assessment of Laryngeal an d Gonadal Responses.
EPA MRID Number 458677-09

Data Requirement::

EPA DP Barcode D288775

EPA MRID 458677-09
EPA Guideline 70-1(Special Study)

Test material: **Purity:** not reported
Common name Atrazine
Chemical name: IUPAC
CAS name 6-chloro-N-ethyl-N'-(1-methylethyl)-1,3,5-triazine-2,4-diamine
CAS No. 1912-24-9
Synonyms
EPA PC Code: 80803

Thomas M Steeger

Primary Reviewer: Thomas M. Steeger, Ph.D., Senior Biologist **Date:** April 4, 2003
Environmental Fate and Effects Division, ERB 4,
U. S. Environmental Protection Agency

Joseph E. Tietge

Secondary Reviewer(s): Joseph E. Tietge, M.S., Research Aquatic Biologist **Date:** April 16, 2003
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Mary J. Frankenberry, Senior Statistician **Date** 5/5/03
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EPA PC Code 080803

Date Evaluation Completed: 05/31/2003

CITATION: Smith, E. E., L. DuPreez, and K. Solomon. 2003. Field exposure of *Xenopus laevis* to atrazine and other triazines in South Africa: feasibility study for site characterization and assessment of laryngeal and gonadal responses. The Institute of Environmental and Human Health, Texas Tech University, Lubbock, Texas (USA) and School of Environmental Sciences and Development, Potchefstroom University for CHE, Potchefstroom, South Africa. Sponsor: Syngenta Crop Protection, Inc., Laboratory Study ID: ECORISK Number SA-01A.

EXECUTIVE SUMMARY:

This study had two objectives, first, to characterize the possible exposure of *Xenopus laevis* larvae, metamorphs and adults to atrazine and related triazines in surface waters in 8 reference (no corn production or atrazine/triazine use) and 8 experimental (exposed habitats in the proximity of corn production and atrazine/triazine use) in the Potdchefstroom region of South Africa. The second objective was to determine whether there were differences in the sex ratios, age, and size classes of *Xenopus laevis* in the reference and exposed sites. Three ponds (C1, C3 and E6) were spring-fed although the majority of sampling sites relied on rain as the primary source of water. Pond surface area in reference sites ranged from 2,000 to 20,500 m^2, while experimental sites ranged from 450 to 68,722 m^2. Watersheds ranged from 170 to 480 ha in reference sites and from 274 to 1,990 ha at experimental sites. Except for site E1 (DO = 6.01 mg/L), dissolved oxygen concentrations were consistently less than 4.5 mg/L. The majority (67%) of the ponds had a maximum depth of less than 133 cm (4.4 feet) and were relatively shallow. It is unclear though why ponds E1and E8 had pH values ranging from 10.1 to 10.2. Atrazine concentrations in reference site water ranged from less than 0.1 µg/L to 0.15 µg/L, while atrazine in corn-growing locations ranged from less than 0.1 µg/L to 1.23 µg/L.

While the proportion of males and females was significantly different at different ponds within the reference and atrazine-exposed sites, the overall mean percentages of males and females in the reference and experimental sites were not different. There was no statistical difference in the size (length and weight) of males frogs collected from reference and experimental sites although females frogs collected from reference sites were significantly larger (length and weight) than females collected at experimental sites. Additionally, frogs from reference ponds tended to be significantly younger than frogs collected from experimental site; however, because an unknown percentage of the frogs collected could not be aged, the actual age structure of the frog populations is uncertain.

Based on the criteria set for the study, *i.e.*, non-growing sites without atrazine, three sites (C1, C3 and C6) were selected as reference sites. Based on the criteria set for exposure sites, *i.e.*, proximity of corn production and known use/presence of atrazine, five sites (E1, E3, E4, E6 and E8) were selected as exposure sites.

I. MATERIALS AND METHODS

GUIDELINE FOLLOWED: Nonguideline Study

COMPLIANCE: Not conducted in accordance with the Good Laboratory Practice as outlined in 40 CFR Part 160, August 19, 1989. However, GLP elements were incorporated into study conduct including:

- Study was conducted in accordance with a written protocol, signed by the study director, principal investigators and sponsor.
- Standard Operating Procedures were developed/available for critical activities.
- Protocol amendments were written and signed by the study director.

A. MATERIALS:

1. Test Material Atrazine

Description: Not reported

Lot No./Batch No. : Not reported

Purity: Not reported

Stability of compound under test conditions: Not reported

Storage conditions of test chemicals: Not reported

2. Test organism:

Species: African clawed frog (*Xenopus laevis*)

Age at test initiation Adults

Weight at study initiation: (mean and range) Not reported

Length at study initiation: (mean and range) Not reported

Source: Adult *X. laevis* were field-collected in two areas (non-corn growing areas and corn growing areas) in the vicinity of Potchefstrooom, South Africa, using traps baited with liver and meat scraps.

B. STUDY DESIGN:

Objective:

1. To characterize the possible exposure of *Xenopus laevis* larvae, metamorphs and adults to atrazine and related triazines in surface waters in reference (no corn production or atrazine/triazine use) and experimental (exposed habitats in the proximity of corn production and atrazine/triazine use) in the Potdchefstroom region of South Africa.

2. To determine whether there are differences in the sex ratios, age, and size classes of *Xenopus laevis* in the reference and exposed sites.

1. Experimental Conditions

Experimental field exposed sites were selected based on the presence of *X. laevis*, closeness of corn production as well as previous and planned use of atrazine and terbuthylazine (2-*tert*-butylamino-4-chloro-6-ethylamino-s-triazine). Reference sites were selected based on the presence of *X. laevis*, lack of corn production, and absence of atrazine and tertbutlyazine. Eight experimental and eight reference sites were evaluated. Water samples (2-L) were analyzed for atrazine.

A total of 8 sites in two adjacent regions (Viljoenskroon corn growing region = E; non-corn growing region = C) were sampled in the vicinity of Potchefstroom, South Africa.

Compounds of interest included atrazine, its metabolites desethylated atrazine (DEA), desisopropyl atrazine (DIA), diaminochlorotriazine (DACT), and terbutylazine plus simazine and acetochlor.

Frogs were collected in 10 baited traps and harvested after two days and transported back to the laboratory. At the lab, frogs were checked for recapture, sexed, weighed and the snout-vent length was measured. Two digits of the longest toe of the right hind foot were removed and fixed in Bouin's solution [for later age analysis]. Frogs were cryo-branded with a number corresponding to the site; date of recapture was indicated by the angle (orientation) of the brand number.

Population size was estimated using the method of Donnelley and Guyer (1994).

II. RESULTS and DISCUSSION: [All results discussed in this section and the next are those reported by the study authors. Although supplemental data are typically used in a qualitative manner only, EFED verified spreadsheet data and ran basic statistical analyses on the major study parameters. See attached appendix. If results appeared to differ in any substantive way, the difference was reported in the text below.]

The morphoedaphic characteristics of the sampling sites are presented in **Table 1**. Three ponds (C1, C3 and E6) were fountain (spring) fed; however, the majority of sampling sites relied on rain as the primary source of water. Pond surface area in the reference sites ranged from 2,000 to 20,500 m^2 (0.49 - 5.07 acres), while experimental sites ranged from 450 to 68,722 m^2 (0.11 - 16.98 acres). Watersheds ranged from 170 to 480 ha (420 to 1,186 acres) in reference sites and from 274 to 1,990 ha (677 to 9.7 acres) at experimental sites. Except for site E1 (DO = 6.01 mg/L), dissolved oxygen concentrations were consistently less than 4.5 mg/L.

The majority (67%) of the ponds had a maximum depth of less than 133 cm (4.4 feet) and were thus relatively shallow.

Atrazine concentration in reference site water ranged from less than 0.1 µg/L to 0.15 µg/L while atrazine in corn-growing locations ranged from less than 0.1 µg/L to 1.23 µg/L (**Table 2**). Based on the criteria set for the study, *i.e.*, non-growing site without atrazine, cites C1, C3 and C6 were selected as reference sites. Based on the criteria set for exposure sites, *i.e.*, proximity of corn production and known use/presence of atrazine, sites E1, E3, E4, E5, E6 and E8 were selected as exposure sites.

On average, males made up 45% of the frogs collected from reference sites, while they comprised 41% of the frogs collected from exposure sites (**Table 3**). There was no significant difference in sex ratios between non-corn growing sites and corn-growing sites. The total number of frogs sexed at reference sites ranged from 16% to 42% while the number sexed from corn-growing sites ranged from 16 to 98%; there was no statistical difference in the percentage of frogs sexed at each site (ANOVA; $p = 0.0898$).

Based on a mark (toe-clip) and recapture study, the estimated population size of frogs in each pond was determined (**Table 4**). Based on this number and the surface area of the pond, the density of the frogs (number per m^2) ranged from 0.04 to 0.18 in reference sites and from 0.01 to 0.81 in corn-growing sites.

Mean weight (**Table 5**) of male frogs from reference sites (21.7 ± 6.59 g) was similar to weights of male frogs collected from experimental sites (17.2 ± 2.52 g). Females were significantly heavier than males in both reference (ANOVA, $p < 0.022$) and experimental sites (ANOVA, $p < 0.0003$); average weight of females collected from reference sites (39.2 ± 5.23 g) was significantly higher (ANOVA, $p < 0.0102$) than the average weight of females collected from experimental sites (27.9± 4.26 g)

Mean snout-vent length (**Table 6**)of male frogs from reference sites (57.1 ± 5.14 cm) was similar to lengths of male frogs collected from experimental sites (53.12 ± 2.93). Females were significantly larger than males in both reference (ANOVA, $p < 0.0296$) and experimental sites (ANOVA, $p < 0.0009$); contrary to what the study authors report, average lengths of females collected from reference sites (68.43 ± 2.94 cm) were significantly larger (ANOVA, $p < 0.0439$) than the average length of females collected from experimental sites (62.25 ± 3.79 cm).

Average age of frogs collected (**Table 7**) ranged from 1.5 to 2.6 years. The oldest frog examined (8 years) was from experimental pond E1. Except for ponds C1, E4 and E8, the largest age group was Year 1. However, in C1 and E8 the largest age class was Year 2,and in pond E4 the largest age class was Year 3. Ponds C1 and E4 also had the largest percentage of frogs over 2 years-old with 45% and 53% of the total sampled in ponds _, respectively. There were no significant differences in average ages between reference and experimental site ponds according to the study authors; however, based on EFED's analysis (see attached SAS output), frogs from reference ponds tended to be significantly (ANOVA, $p < 0.0007$) younger (1.77 ± 1.02 years) than frogs collected from experimental sites (2.14 ± 1.25 year). It is important to note though that the study authors were not able to age all of the frogs collected; however, they did not provide any data on the number of frogs that they were not able to age. As a result, the age distribution for this study is uncertain.

Table 1. Morphoedaphic characteristics of control (non-corn growing) and experimental (corn-growing) ponds.

Parameter	Control (non-corn) Sites			Experimental (corn-growing) Sites					
	C1	C3	C6	E1	E3	E4	E5	E6	E8
Surface area	20,500 m^2	2,000 m^2	14,860 m^2	7,406 m^2	46,076 m^2	2,400 m^2	450 m^2	68,722 m^2	2,400 m^2
Watershed area	244 ha	170 ha	480 ha	1,990 ha	1,046 ha	448 ha	274 ha	515 ha	1,100 ha
Deepest Point	261 cm	38.5 cm	104 cm	118 cm	133 cm	44 cm	67cm	370 cm	175 cm
pH	8.3	7.3	8.2	10.1	9.1	8.2	8.0	8.4	10.2
DO	3.39 mg/L	3.39 mg/L	3.14 mg/L	6.01 mg/L	3.96 mg/L	4.51 mg/L	4.05 mg/L	3.88 mg/L	4.35 mg/L
Secchi depth	11.5 cm	32 cm	6.5 cm	118 cm	133 cm	44 cm	67 cm	207 cm	175 cm
Conductivity	2.61 µS/m	4.77 µS/m	8.6 µS/m	186 µS/m	144 µS/m	116.7 µS/m	134.6 µS/m	57.5 µS/m	54.0 µS/m

Table 2. Atrazine concentrations in water collected from non-corn growing "reference" (CI - C8) and corn-growing "experimental" (E1 - E8) sites in South Africa.

Site	Potch Value (µg/L)	Syngenta Value (µg/L)
C1	< 0.10	BROKEN
C2	0.15	0.13
C3	< 0.10	< 0.10
C4	0.11	NOT ANALYZED
C5	< 0.10	NOT ANALYZED
C6	< 0.10	NOT ANALYZED
C7	< 0.10	NOT ANALYZED
C8	< 0.10	NOT ANALYZED
E1	1.23	0.96
E2	< 0.10	< 0.10
E3	0.32	0.19
E4	0.12	BROKEN
E5	< 0.10	< 0.10
E6	0.68	1.1
E7	0.51	NOT ANALYZED
E8	0.84	NOT ANALYZED

Table 3. Sex ratio and total[1] number of frogs collected from non-corn growing (C1 - C6) and corn-growing (E1 - E8) sites in South Africa.

Sex	Reference C1	Reference C3	Reference C6	Experimental E1	Experimental E3	Experimental E4	Experimental E5	Experimental E6	Experimental E8
Juvenile	0	3	1	4	0	3	0	1	6
Male	39	52	41	51	46	22	15	34	45
Female	61	45	58	45	54	43	51	65	49
Total	289	229	621	118	620	84	87	195	96

[1] total number of frogs collected during three trapping efforts used to estimate frog population number through mark and recapture. This number does **not** represent the total number of males and females used in estimating the sex ratios.

Table 4. Estimated population size of frogs at reference (non-corn growing) and experimental (corn-growing) sites in South Africa.

	Reference C1	Reference C3	Reference C6	Experimental E1	Experimental E3	Experimental E4	Experimental E5	Experimental E6	Experimental E8
Number	713	360	950	370	1218	354	364	909	216
Surface area (m^2)	20500	2000	14860	7406	46076	24000	450	68722	2400
Density ($\#/m^2$)	0.035	0.180	0.064	0.05	0.026	0.148	0.809	0.013	0.09

Table 5. Mean weight (gms) of frogs collected from reference (non-corn growing) and experimental (corn-growing) sites in South Africa.

Sex	Reference C1	Reference C3	Reference C6	Experimental E1	Experimental E3	Experimental E4	Experimental E5	Experimental E6	Experimental E8
Juvenile		3.8	6.4	4.2	--	4.0	--	6.8	6.0
Male	18.6	17.3	29.3	14.5	15.4	21.1	15.3	18.5	18.1
Female	45.0	34.9	37.6	20.8	29.6	33.8	28.3	28.6	26.5

Table 6. Mean snout-vent length (mm) of frogs collected from non-corn growing (Reference) and corn-growing sites.

	Reference			Experimental					
Sex	**C1**	**C3**	**C6**	**E1**	**E3**	**E4**	**E5**	**E6**	**E8**
Juvenile	--	32.3	37.5	33.8	--	32.7	--	37.7	36.3
Male	55.7	52.8	62.8	50.3	50.8	57.9	51.1	54.3	54.3
Female	71.6	65.9	67.9	57.1	60.6	68.7	62.0	63.1	62.0

Table 7. Age structure of African clawed frogs (*Xenopus laevis*) in reference (C1 - C6) and experimental (E1 - E8) ponds based on mark and recapture (3 efforts) study.

	Reference			Experimental				
Years	**C1**	**C3**	**C6**	**E1**	**E3**	**E4**	**E6**	**E8**
1	9	43	49	34	24	14	21	14
2	22	16	14	15	12	11	21	27
3	13	3	6	9	4	18	9	6
4	10	2	1	6	2	8	4	5
5	1	0	1	3	0	1	4	2
6	1	0	0	1	0	1	1	1
7	0	0	0	0	0	0	0	0
8	0	0	0	1	0	0	0	0
Total Number	56	64	71	69	42	53	60	55
Mean Age	2.55	1.44	1.46	2.09	1.62	2.51	2.20	2.22

D. <u>VERIFICATION OF STATISTICAL RESULTS</u>: See attached Statistical Analysis System (SAS Release 8.01, Cary, North Carolina)

E. STUDY DEFICIENCIES:

Study authors were unable to age all of the frogs collected. The exact age structure and population size are uncertain.

F. REVIEWER'S COMMENTS:

The pH of all the ponds were alkaline, especially ponds E1 and E8 which were as high as 10.2. The study authors have suggested that the surrounding corn fields were treated with agricultural limestone (personal communication Allan Hosmer, Syngenta, April 2003); however, agricultural limestone (calcium/magnesium carbonate) is not likely to raise pond water to pH values above 8.0. It is possible that farmers may have treated surrounding fields with hydrated or slaked lime.

The study's inability to age all of the frogs collected renders it difficult to determine the age and population size of the tested animals .

This study was designed as a general survey to determine the utility of the study sites to serve as atrazine-exposed versus reference sites. After these sites are selected a more detailed assessment of the effects of atrazine and other triazines on the gonadal development, blood steroid, and gonadal aromatase activity of African clawed frogs will be studied.

G. CONCLUSIONS:

Three ponds (C1, C3 and E6)were spring-fed; however, the majority of sampling sites relied on rain as the primary source of water. Pond surface area in reference sites ranged from 2,000 to 20,500 m^2 while experimental sites ranged from 450 to 68,722 m^2. Watersheds ranged from 170 to 480 ha in reference sites and from 274 to 1,990 ha at experimental sites. Except for site E1 (DO = 6.01 mg/L), dissolved oxygen concentrations were consistently less than 4.5 mg/L. The majority (67%) of the ponds had a maximum depth of less than 133 cm (4.4 feet) and were thus relatively shallow. It is unclear though why ponds E1and E8 had pH values ranging from 10.1 to 10.2. Atrazine concentration in reference site water ranged from less than 0.1 µg/L to 0.15 µg/L while atrazine in corn-growing locations ranged from less than 0.1 µg/L to 1.23 µg/L.

While the proportion of males and females was significantly different across sites, the mean percentage of males and females in the reference and experimental sites was not different. There was no statistical difference in the size (length and weight) of male frogs collected from reference and experimental sites; however female frogs collected from reference sites were significantly larger (length and weight) than females collected at experimental sites. Additionally, frogs from reference ponds tended to be significantly younger than frogs collected from experimental site; however, given that an unknown percentage of the frogs collected could not be aged, the actual age structure of the frog populations is uncertain..

Based on the criteria set for the study, *i.e.*, non-growing site without atrazine, 3 sites (C1, C3 and C6) were selected as reference sites. Based on the criteria set for exposure sites, *i.e.*, proximity of corn production and known use/presence of atrazine, 5 sites (E1, E3, E4, E6 and E8) were selected as exposure sites. It should be noted that C1 and C3 are both cattle farms while C6 is located in the security buffer zone outside of an explosive factory.

MEAN WEIGHTS AND LENGTHS OF MALE XENOPUS COLLECTED FROM REFERENCE (C) AND EXPERIMENTAL (E) PO 48

Obs	TREAT	_TYPE_	_FREQ_	MEAN_W	MEAN_L	SD_W	SD_L
1	C	0	3	21.7333	57.1000	6.58508	5.14490
2	E	0	6	17.1500	53.1167	2.52329	2.93422

53
1

ANOVA FOR MALE WEIGHT

The ANOVA Procedure

Class Level Information

Class	Levels	Values
POND	2	C E

Number of observations 9

Dependent Variable: WEIGHT

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	1	42.0138889	42.0138889	2.48	0.1593
Error	7	118.5616667	16.9373810		
Corrected Total	8	160.5755556			

R-Square	Coeff Var	Root MSE	WEIGHT Mean
0.261646	22.03423	4.115505	18.67778

Source	DF	Anova SS	Mean Square	F Value	Pr > F
POND	1	42.01388889	42.01388889	2.48	0.1593

Levene's Test for Homogeneity of WEIGHT Variance
ANOVA of Squared Deviations from Group Means

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
POND	1	1114.2	1114.2	5.56	0.0505
Error	7	1402.7	200.4		

Bartlett's Test for Homogeneity of WEIGHT Variance

Source	DF	Chi-Square	Pr > ChiSq
POND	1	2.5401	0.1110

NOTE: This test controls the Type I experimentwise error for comparisons of all treatments against a control.

Alpha	0.05
Error Degrees of Freedom	7
Error Mean Square	16.93738
Critical Value of Dunnett's t	2.36463

Comparisons significant at the 0.05 level are indicated by ***.

POND Comparison	Difference Between Means	Simultaneous 95% Confidence Limits	
E - C	-4.583	-11.465	2.298

NONPARAMETRIC COMPARISON OF MALE FROG WEIGHT BETWEEN PONDS 5

The NPAR1WAY Procedure

Wilcoxon Scores (Rank Sums) for Variable WEIGHT
Classified by Variable POND

POND	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
C	3	20.0	15.0	3.872983	6.666667
E	6	25.0	30.0	3.872983	4.166667

Wilcoxon Two-Sample Test

Statistic	20.0000
Normal Approximation	
Z	1.1619
One-Sided Pr > Z	0.1226
Two-Sided Pr > |Z|	0.2453
t Approximation	
One-Sided Pr > Z	0.1394
Two-Sided Pr > |Z|	0.2788

Z includes a continuity correction of 0.5.

Kruskal-Wallis Test

Chi-Square	1.6667
DF	1
Pr > Chi-Square	0.1967

Median Scores (Number of Points Above Median) for Variable WEIGHT
Classified by Variable POND

POND	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
C	3	2.0	1.333333	0.745356	0.666667
E	6	2.0	2.666667	0.745356	0.333333

Median Two-Sample Test

Statistic	2.0000
Z	0.8944
One-Sided Pr > Z	0.1855
Two-Sided Pr > |Z|	0.3711

Median One-Way Analysis

Chi-Square	0.8000
DF	1
Pr > Chi-Square	0.3711

ANOVA FOR MALE LENGTH

The ANOVA Procedure

Class Level Information

Class	Levels	Values
POND	2	C E

Number of observations 9

Dependent Variable: LENGTH

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	1	31.7338889	31.7338889	2.31	0.1720
Error	7	95.9883333	13.7126190		
Corrected Total	8	127.7222222			

R-Square	Coeff Var	Root MSE	LENGTH Mean
0.248460	6.801530	3.703055	54.44444

Source	DF	Anova SS	Mean Square	F Value	Pr > F
POND	1	31.73388889	31.73388889	2.31	0.1720

Levene's Test for Homogeneity of LENGTH Variance
ANOVA of Squared Deviations from Group Means

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
POND	1	219.3	219.3	1.93	0.2070
Error	7	794.0	113.4		

Bartlett's Test for Homogeneity of LENGTH Variance

Source	DF	Chi-Square	Pr > ChiSq
POND	1	0.8533	0.3556

Dunnett's t Tests for LENGTH

NOTE: This test controls the Type I experimentwise error for comparisons of all treatments against a control.

Alpha	0.05
Error Degrees of Freedom	7
Error Mean Square	13.71262
Critical Value of Dunnett's t	2.36463

Comparisons significant at the 0.05 level are indicated by ***.

POND Comparison	Difference Between Means	Simultaneous 95% Confidence Limits	
E - C	-3.983	-10.175	2.208

NONPARAMETRIC COMPARISON OF MALE FROG LENGTH BETWEEN PONDS 11

The NPAR1WAY Procedure

Wilcoxon Scores (Rank Sums) for Variable LENGTH
Classified by Variable POND

POND	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
C	3	20.0	15.0	3.856812	6.666667
E	6	25.0	30.0	3.856812	4.166667

Average scores were used for ties.

Wilcoxon Two-Sample Test

Statistic	20.0000
Normal Approximation	
Z	1.1668
One-Sided Pr > Z	0.1217
Two-Sided Pr > |Z|	0.2433
t Approximation	
One-Sided Pr > Z	0.1385
Two-Sided Pr > |Z|	0.2769

Z includes a continuity correction of 0.5.

Kruskal-Wallis Test

Chi-Square	1.6807
DF	1
Pr > Chi-Square	0.1948

Median Scores (Number of Points Above Median) for Variable LENGTH
Classified by Variable POND

POND	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
C	3	2.0	1.333333	0.656167	0.666667
E	6	2.0	2.666667	0.656167	0.333333

Average scores were used for ties.

Median Two-Sample Test

Statistic	2.0000
Z	1.0160
One-Sided Pr > Z	0.1548
Two-Sided Pr > |Z|	0.3096

Median One-Way Analysis

Chi-Square	1.0323
DF	1
Pr > Chi-Square	0.3096

ANOVA FOR FEMALE WEIGHT

The ANOVA Procedure

Class Level Information

Class	Levels	Values
POND	2	C E

Number of observations 9

Dependent Variable: WEIGHT

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	1	239.0755556	239.0755556	10.89	0.0131
Error	7	153.6200000	21.9457143		
Corrected Total	8	392.6955556			

R-Square	Coeff Var	Root MSE	WEIGHT Mean
0.608806	14.83520	4.684625	31.57778

Source	DF	Anova SS	Mean Square	F Value	Pr > F
POND	1	239.0755556	239.0755556	10.89	0.0131

Levene's Test for Homogeneity of WEIGHT Variance
ANOVA of Squared Deviations from Group Means

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
POND	1	68.4450	68.4450	0.16	0.7048
Error	7	3074.0	439.1		

Bartlett's Test for Homogeneity of WEIGHT Variance

Source	DF	Chi-Square	Pr > ChiSq
POND	1	0.1954	0.6585

NOTE: This test controls the Type I experimentwise error for comparisons of all treatments against a control.

Alpha	0.05
Error Degrees of Freedom	7
Error Mean Square	21.94571
Critical Value of Dunnett's t	2.36463

Comparisons significant at the 0.05 level are indicated by ***.

POND Comparison	Difference Between Means	Simultaneous 95% Confidence Limits		
E - C	-10.933	-18.766	-3.100	***

NONPARAMETRIC COMPARISON OF FEMALE FROG WEIGHT BETWEEN PONDS 17

The NPAR1WAY Procedure

Wilcoxon Scores (Rank Sums) for Variable WEIGHT
Classified by Variable POND

POND	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
C	3	24.0	15.0	3.872983	8.00
E	6	21.0	30.0	3.872983	3.50

Wilcoxon Two-Sample Test

Statistic	24.0000
Normal Approximation	
Z	2.1947
One-Sided Pr > Z	0.0141
Two-Sided Pr > |Z|	0.0282
t Approximation	
One-Sided Pr > Z	0.0297
Two-Sided Pr > |Z|	0.0595

Z includes a continuity correction of 0.5.

Kruskal-Wallis Test

Chi-Square	5.4000
DF	1
Pr > Chi-Square	0.0201

Median Scores (Number of Points Above Median) for Variable WEIGHT
Classified by Variable POND

POND	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
C	3	3.0	1.333333	0.745356	1.000000
E	6	1.0	2.666667	0.745356	0.166667

Median Two-Sample Test

Statistic	3.0000
Z	2.2361
One-Sided Pr > Z	0.0127
Two-Sided Pr > |Z|	0.0253

Median One-Way Analysis

Chi-Square	5.0000
DF	1
Pr > Chi-Square	0.0253

ANOVA FOR FEMALE LENGTH

The ANOVA Procedure

Class Level Information

Class	Levels	Values
POND	2	C E

Number of observations 9

Dependent Variable: LENGTH

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	1	76.4672222	76.4672222	6.02	0.0439
Error	7	88.9416667	12.7059524		
Corrected Total	8	165.4088889			

R-Square	Coeff Var	Root MSE	LENGTH Mean
0.462292	5.542652	3.564541	64.31111

Source	DF	Anova SS	Mean Square	F Value	Pr > F
POND	1	76.46722222	76.46722222	6.02	0.0439

Levene's Test for Homogeneity of LENGTH Variance
ANOVA of Squared Deviations from Group Means

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
POND	1	76.8869	76.8869	0.33	0.5841
Error	7	1635.0	233.6		

Bartlett's Test for Homogeneity of LENGTH Variance

Source	DF	Chi-Square	Pr > ChiSq
POND	1	0.1439	0.7044

Dunnett's t Tests for LENGTH

NOTE: This test controls the Type I experimentwise error for comparisons of all treatments against a control.

Alpha	0.05
Error Degrees of Freedom	7
Error Mean Square	12.70595
Critical Value of Dunnett's t	2.36463

Comparisons significant at the 0.05 level are indicated by ***.

POND Comparison	Difference Between Means	Simultaneous 95% Confidence Limits		
E - C	-6.183	-12.143	-0.223	***

The NPAR1WAY Procedure

Wilcoxon Scores (Rank Sums) for Variable LENGTH
Classified by Variable POND

POND	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
C	3	22.0	15.0	3.856812	7.333333
E	6	23.0	30.0	3.856812	3.833333

Average scores were used for ties.

Wilcoxon Two-Sample Test

Statistic	22.0000
Normal Approximation	
Z	1.6853
One-Sided Pr > Z	0.0460
Two-Sided Pr > |Z|	0.0919
t Approximation	
One-Sided Pr > Z	0.0652
Two-Sided Pr > |Z|	0.1304

Z includes a continuity correction of 0.5.

Kruskal-Wallis Test

Chi-Square	3.2941
DF	1
Pr > Chi-Square	0.0695

Median Scores (Number of Points Above Median) for Variable LENGTH
Classified by Variable POND

POND	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
C	3	3.0	1.333333	0.745356	1.000000
E	6	1.0	2.666667	0.745356	0.166667

Average scores were used for ties.

Median Two-Sample Test

Statistic	3.0000
Z	2.2361
One-Sided Pr > Z	0.0127
Two-Sided Pr > |Z|	0.0253

Median One-Way Analysis

Chi-Square	5.0000
DF	1
Pr > Chi-Square	0.0253

COMPARISON OF MALE AND FEMALE WEIGHT BY SAMPLING AREA 25

-- POND=C --

The ANOVA Procedure

Class Level Information

Class	Levels	Values
SEX	2	FEMA MALE

Number of observations 6

Dependent Variable: WEIGHT

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	1	440.3266667	440.3266667	11.77	0.0265
Error	4	149.6333333	37.4083333		
Corrected Total	5	589.9600000			

R-Square	Coeff Var	Root MSE	WEIGHT Mean
0.746367	20.18559	6.116235	30.30000

Source	DF	Anova SS	Mean Square	F Value	Pr > F
SEX	1	440.3266667	440.3266667	11.77	0.0265

Levene's Test for Homogeneity of WEIGHT Variance
ANOVA of Squared Deviations from Group Means

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
SEX	1	94.5654	94.5654	0.20	0.6796
Error	4	1913.1	478.3		

Bartlett's Test for Homogeneity of WEIGHT Variance

Source	DF	Chi-Square	Pr > ChiSq
SEX	1	0.0411	0.8394

Dunnett's t Tests for WEIGHT

NOTE: This test controls the Type I experimentwise error for comparisons of all treatments against a control.

Alpha	0.05
Error Degrees of Freedom	4
Error Mean Square	37.40833
Critical Value of Dunnett's t	2.77630
Minimum Significant Difference	13.865

Comparisons significant at the 0.05 level are indicated by ***.

SEX Comparison	Difference Between Means	Simultaneous 95% Confidence Limits		
MALE - FEMA	-17.133	-30.998	-3.269	***

COMPARISON OF MALE AND FEMALE WEIGHT BY SAMPLING AREA 29

--- POND=E ---

The ANOVA Procedure

Class Level Information

Class	Levels	Values
SEX	2	FEMA MALE

Number of observations 12

Dependent Variable: WEIGHT

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	1	348.8408333	348.8408333	28.47	0.0003
Error	10	122.5483333	12.2548333		
Corrected Total	11	471.3891667			

R-Square	Coeff Var	Root MSE	WEIGHT Mean
0.740027	15.52987	3.500690	22.54167

Source	DF	Anova SS	Mean Square	F Value	Pr > F
SEX	1	348.8408333	348.8408333	28.47	0.0003

Levene's Test for Homogeneity of WEIGHT Variance
ANOVA of Squared Deviations from Group Means

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
SEX	1	288.9	288.9	1.13	0.3134
Error	10	2563.5	256.4		

Bartlett's Test for Homogeneity of WEIGHT Variance

Source	DF	Chi-Square	Pr > ChiSq
SEX	1	1.1929	0.2747

Dunnett's t Tests for WEIGHT

NOTE: This test controls the Type I experimentwise error for comparisons of all treatments against a control.

Alpha	0.05
Error Degrees of Freedom	10
Error Mean Square	12.25483
Critical Value of Dunnett's t	2.22816
Minimum Significant Difference	4.5034

Comparisons significant at the 0.05 level are indicated by ***.

SEX Comparison	Difference Between Means	Simultaneous 95% Confidence Limits		
MALE - FEMA	-10.783	-15.287	-6.280	***

NONPARAMETRIC COMPARISON OF MALE AND FEMALE WEIGHT BY SAMPLING AREA 33

-- POND=C --

The NPAR1WAY Procedure

Wilcoxon Scores (Rank Sums) for Variable WEIGHT
Classified by Variable SEX

SEX	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
FEMA	3	15.0	10.50	2.291288	5.0
MALE	3	6.0	10.50	2.291288	2.0

Wilcoxon Two-Sample Test

Statistic	15.0000
Normal Approximation	
Z	1.7457
One-Sided Pr > Z	0.0404
Two-Sided Pr > |Z|	0.0809
t Approximation	
One-Sided Pr > Z	0.0706
Two-Sided Pr > |Z|	0.1413

Z includes a continuity correction of 0.5.

Kruskal-Wallis Test

Chi-Square	3.8571
DF	1
Pr > Chi-Square	0.0495

Median Scores (Number of Points Above Median) for Variable WEIGHT
Classified by Variable SEX

SEX	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
FEMA	3	3.0	1.50	0.670820	1.0
MALE	3	0.0	1.50	0.670820	0.0

Median Two-Sample Test

Statistic	3.0000
Z	2.2361
One-Sided Pr > Z	0.0127
Two-Sided Pr > |Z|	0.0253

Median One-Way Analysis

Chi-Square	5.0000
DF	1
Pr > Chi-Square	0.0253

NONPARAMETRIC COMPARISON OF MALE AND FEMALE WEIGHT BY SAMPLING AREA 35

--- POND=E ---

The NPAR1WAY Procedure

Wilcoxon Scores (Rank Sums) for Variable WEIGHT
Classified by Variable SEX

SEX	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
FEMA	6	56.0	39.0	6.244998	9.333333
MALE	6	22.0	39.0	6.244998	3.666667

Wilcoxon Two-Sample Test

Statistic	56.0000
Normal Approximation	
Z	2.6421
One-Sided Pr > Z	0.0041
Two-Sided Pr > |Z|	0.0082
t Approximation	
One-Sided Pr > Z	0.0115
Two-Sided Pr > |Z|	0.0229

Z includes a continuity correction of 0.5.

Kruskal-Wallis Test

Chi-Square	7.4103
DF	1
Pr > Chi-Square	0.0065

Median Scores (Number of Points Above Median) for Variable WEIGHT
Classified by Variable SEX

SEX	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
FEMA	6	5.0	3.0	0.904534	0.833333
MALE	6	1.0	3.0	0.904534	0.166667

Median Two-Sample Test

Statistic	5.0000
Z	2.2111
One-Sided Pr > Z	0.0135
Two-Sided Pr > |Z|	0.0270

Median One-Way Analysis

Chi-Square	4.8889
DF	1
Pr > Chi-Square	0.0270

COMPARISON OF MALE AND FEMALE LENGTH BY SAMPLING AREA 37

-- POND=C --

The ANOVA Procedure

Class Level Information

Class	Levels	Values
SEX	2	FEMA MALE

Number of observations 6

Dependent Variable: LENGTH

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	1	192.6666667	192.6666667	10.98	0.0296
Error	4	70.1866667	17.5466667		
Corrected Total	5	262.8533333			

R-Square	Coeff Var	Root MSE	LENGTH Mean
0.732982	6.673724	4.188874	62.76667

Source	DF	Anova SS	Mean Square	F Value	Pr > F
SEX	1	192.6666667	192.6666667	10.98	0.0296

Levene's Test for Homogeneity of LENGTH Variance
ANOVA of Squared Deviations from Group Means

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
SEX	1	212.3	212.3	1.64	0.2691
Error	4	516.7	129.2		

Bartlett's Test for Homogeneity of LENGTH Variance

Source	DF	Chi-Square	Pr > ChiSq
SEX	1	0.4788	0.4890

Dunnett's t Tests for LENGTH

NOTE: This test controls the Type I experimentwise error for comparisons of all treatments against a control.

Alpha	0.05
Error Degrees of Freedom	4
Error Mean Square	17.54667
Critical Value of Dunnett's t	2.77630
Minimum Significant Difference	9.4955

Comparisons significant at the 0.05 level are indicated by ***.

SEX Comparison	Difference Between Means	Simultaneous 95% Confidence Limits		
MALE - FEMA	-11.333	-20.829	-1.838	***

COMPARISON OF MALE AND FEMALE LENGTH BY SAMPLING AREA 41

-- POND=E --

The ANOVA Procedure

Class Level Information

Class	Levels	Values
SEX	2	FEMA MALE

Number of observations 12

Dependent Variable: LENGTH

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	1	250.2533333	250.2533333	21.81	0.0009
Error	10	114.7433333	11.4743333		
Corrected Total	11	364.9966667			

R-Square	Coeff Var	Root MSE	LENGTH Mean
0.685632	5.872370	3.387379	57.68333

Source	DF	Anova SS	Mean Square	F Value	Pr > F
SEX	1	250.2533333	250.2533333	21.81	0.0009

Levene's Test for Homogeneity of LENGTH Variance
ANOVA of Squared Deviations from Group Means

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
SEX	1	68.3860	68.3860	0.36	0.5631
Error	10	1912.3	191.2		

Bartlett's Test for Homogeneity of LENGTH Variance

Source	DF	Chi-Square	Pr > ChiSq
SEX	1	0.2925	0.5886

Dunnett's t Tests for LENGTH

NOTE: This test controls the Type I experimentwise error for comparisons of all treatments against a control.

Alpha	0.05
Error Degrees of Freedom	10
Error Mean Square	11.47433
Critical Value of Dunnett's t	2.22816
Minimum Significant Difference	4.3576

Comparisons significant at the 0.05 level are indicated by ***.

SEX Comparison	Difference Between Means	Simultaneous 95% Confidence Limits		
MALE - FEMA	-9.133	-13.491	-4.776	***

NONPARAMETRIC COMPARISON OF MALE AND FEMALE LENGTH BY SAMPLING AREA 45

-- POND=C --

The NPAR1WAY Procedure

Wilcoxon Scores (Rank Sums) for Variable LENGTH
Classified by Variable SEX

SEX	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
FEMA	3	15.0	10.50	2.291288	5.0
MALE	3	6.0	10.50	2.291288	2.0

Wilcoxon Two-Sample Test

Statistic	15.0000
Normal Approximation	
Z	1.7457
One-Sided Pr > Z	0.0404
Two-Sided Pr > |Z|	0.0809
t Approximation	
One-Sided Pr > Z	0.0706
Two-Sided Pr > |Z|	0.1413

Z includes a continuity correction of 0.5.

Kruskal-Wallis Test

Chi-Square	3.8571
DF	1
Pr > Chi-Square	0.0495

The NPAR1WAY Procedure

Median Scores (Number of Points Above Median) for Variable LENGTH
Classified by Variable SEX

SEX	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
FEMA	3	3.0	1.50	0.670820	1.0
MALE	3	0.0	1.50	0.670820	0.0

Median Two-Sample Test

Statistic	3.0000
Z	2.2361
One-Sided Pr > Z	0.0127
Two-Sided Pr > |Z|	0.0253

Median One-Way Analysis

Chi-Square	5.0000
DF	1
Pr > Chi-Square	0.0253

NONPARAMETRIC COMPARISON OF MALE AND FEMALE LENGTH BY SAMPLING AREA 47

-- POND=E --

The NPAR1WAY Procedure

Wilcoxon Scores (Rank Sums) for Variable LENGTH
Classified by Variable SEX

SEX	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
FEMA	6	56.0	39.0	6.223124	9.333333
MALE	6	22.0	39.0	6.223124	3.666667

Average scores were used for ties.

Wilcoxon Two-Sample Test

Statistic	56.0000
Normal Approximation	
Z	2.6514
One-Sided Pr > Z	0.0040
Two-Sided Pr > |Z|	0.0080
t Approximation	
One-Sided Pr > Z	0.0113
Two-Sided Pr > |Z|	0.0225

Z includes a continuity correction of 0.5.

Kruskal-Wallis Test

Chi-Square	7.4624
DF	1
Pr > Chi-Square	0.0063

The NPAR1WAY Procedure

Median Scores (Number of Points Above Median) for Variable LENGTH
Classified by Variable SEX

SEX	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
FEMA	6	5.0	3.0	0.904534	0.833333
MALE	6	1.0	3.0	0.904534	0.166667

Average scores were used for ties.

Median Two-Sample Test

Statistic	5.0000
Z	2.2111
One-Sided Pr > Z	0.0135
Two-Sided Pr > |Z|	0.0270

Median One-Way Analysis

Chi-Square	4.8889
DF	1
Pr > Chi-Square	0.0270

Data Evaluation Report on Gonadal and Laryngeal Responses to Field Exposure of *Xenopus laevis* to Atrazine in Areas of Corn Production in South Africa

EPA MRID Number 458677-10

Data Requirement:

EPA DP Barcode D288775

EPA MRID 458677-10
EPA Guideline 70-1(Special Study)

Test material: **Purity:** not reported
Common name: Atrazine
Chemical name: IUPAC
CAS name 6-chloro-N-ethyl-N'-(1-methylethyl)-1,3,5-triazine-2,4-diamine
CAS No. 1912-24-9
synonyms
EPA PC Code: 80803

Thomas M Steeger

Primary Reviewer: Thomas M. Steeger, Ph.D., Senior Biologist **Date:** April 12, 2003
Environmental Fate and Effects Division, ERB 4,
U. S. Environmental Protection Agency

Joseph E. Tietge 4/16/03

Secondary Reviewer(s): Joseph E. Tietge, M.S., Research Aquatic Biologist **Date:**
Mid-Continent Ecology Division,
National Health and Environmental Effects Research Laboratory (Duluth),
U. S. Environmental Protection Agency

Stephanie R. Irene

Stephanie Irene, Ph.D., Senior Advisor **Date:** 4/29/03
Environmental Fate and Effects Division, ERB 3,
U. S. Environmental Protection Agency

Mary Frankenberry

Mary J. Frankenberry, Statistician **Date:** 4/28/03
Environmental Fate and Effects Division, ERB 3,
U. S. Environmental Protection Agency

EPA PC Code 080803

Date Evaluation Completed: 05/31/2003

CITATION: Smith, E. E., L. DuPreez, and K. Solomon. 2003. Gonadal and laryngeal responses to field exposure of *Xenopus laevis* to atrazine in areas of corn production in South Africa. The Institute of Environmental and Human Health, Texas Tech University, Lubbock, TX and School of Environmental Sciences and Development, Potchefstroom University for CHE, Potchefstroom, South Africa. Sponsor: Syngenta Crop Protection, Inc., Laboratory Study ID: ECORISK Number SA-01C.

EXECUTIVE SUMMARY:

This report is part of a multiphase study examining the effects of atrazine on African clawed frogs (*Xenopus laevis*) in their native habitat (South Africa). This phase of the study summarizes the body weights, lengths and condition index (weight/length) of both male and female collected from reference site (no corn grown and no atrazine/triazine use) and experimental site (corn grown and atrazine/triazines used) ponds. The study also examined several aspects of gonadal growth (testis weight, gonadosomatic index) and development (incidence of deformities, cellular morphology) in frog testes collected from the two sites to determine whether atrazine exposure had affected these measurement endpoints.

While males were smaller than females at both study sites, there was no difference in weight or length of males or females collected at reference or experimental sites. Condition indices for males and females did not differ between reference and experimental collection sites. However, the mean testes weight from reference ponds (0.037 ± 0.021 mg) was statistically different from mean weight of testes (0.046 ± 0.018 mg) from frogs collected at experimental sites. GSI was significantly different for males collected from reference (0.13 ± 0.04) compared to males collected from experimental sites (0.17 ± 0.04).

There were no statistical differences in the cellular morphology of testes collected at reference sites from those collected at experimental site. The incidence of external abnormalities (missing limbs) was 1% across all animals collected (reference and experimental) and was attributed to the predatory effects of turtles. The incidence of testicular oocytes was 3% in males collected from reference ponds and 2% in experimental ponds. The study claims that laryngeal somatic index for males and females from reference and experimental sites were not significantly different ($p > 0.05$); however, no raw data were provided to verify these observations.

Although not discussed at length in this phase of the study, the interpretation of these data is complicated by the fact that atrazine, its degradates and other triazines were detected at significant levels in reference ponds. Additionally, at some sites, frogs were collected over a protracted period of time (6 months), rendering GSI a questionable metric. This study provides evidence that under the conditions studied, the incidence of testicular oocytes in Xenopus laevis is roughly 2 to 3%. Because of the presence of atrazine in both sampling areas, a regression-based analysis would be more appropriate than hypothesis-testing based analysis. Unfortunately, though, the study is not designed for regression analysis.

I. MATERIALS AND METHODS

GUIDELINE FOLLOWED: Nonguideline Study

COMPLIANCE: Not conducted under full GLP; however, most practices as defined by 40 CFR Part 160, August 19, 1989 were established for this study, including but not limited to:

- Written, authorized protocol
- Written, authorized Standard Operating Procedures for all key procedures.
- Organization and personnel were sufficient in terms of number, education, training and experience.
- Facilities were of suitable size and construction
- Equipment used was of appropriate design and adequate capacity.
- Test material identity, strength, purity and composition were characterized
- Independent QA inspections of raw data and final report were conducted.
- Interim Report was written
- Raw data, documentation, records, protocols, and final phase report will be archived.

A. MATERIALS:

1. Test Material Atrazine

Description: Not reported

Lot No./Batch No. : Not reported

Purity: Not reported
Stability of compound under test conditions: Not reported

Storage conditions of test chemicals: Not reported

2. Test organism:

Species: African clawed frog (*Xenopus laevis*)

Age at test initiation: : Adults
Weight at study initiation (mean and range): Not reported
Length at study initiation (mean and range): Not reported

Source: Adult *X. laevis* were field-collected in two areas (non-corn growing areas and corn growing areas) in the vicinity of Potchefstrooom, South Africa, using traps baited with liver and meat scraps.

B. STUDY DESIGN:

Objective:

1. To determine if exposure to triazines used in corn production affects the incidence or extent of morphological changes in the gonads or intersex gonads in the male and female metamorphs and adults of *X. laevis*.
2. To determine whether there are changes in the morphology of the male larynx in metamorphs and adults of *X. laevis* in response to exposures to triazines used in corn production.
3. To determine if there are changes in plasma steroid concentrations of adult *X. laevis* in response to exposures to triazines used in corn production.
4. To determine if other frogs present at the sites show any morphological or other changes in the gonads.
5. To assess feasibility of determining if *X. laevis* in the exposed sites have been subjected to selection pressure.

1. Experimental Conditions

Sampling was conducted in April and May approximately 6 months after atrazine was applied (October / November); water samples to characterize exposure were collected one month prior to frog sampling. Environmental parameters that were measured at each site included habitat description (vegetative cover and water depth). Water samples were analyzed for atrazine, related triazines, other pesticides and metals.

A total of eight sites were sampled in two adjacent regions (Viljoenskroon corn growing region = E; non-corn growing region = C) in the vicinity of Potchefstroom, South Africa.

Compounds of interest included atrazine, its metabolites desethylated atrazine (DEA), desisopropyl atrazine (DIA), diaminochlorotriazine (DACT), plus other herbicides (terbutylazine, simazine and acetochlor).

Frogs were sampled after the rainy season in April and May; however, at atrazine sites E1 and E8, frogs were collected from April to June and from April to September, respectively, due to low sample sizes. The low sample sizes at sites E1 and E8 were attributed to the introduction of sharptooth catfish (*Clarius gariepinus*) into the ponds due to high runoff. Sampling at all other sites occurred once or twice within a "few days." Frogs were collected in 10 baited traps and harvested after two days in "clean water". Afterwards, animals were housed individually in 2-L plastic containers for 48 hours to recover from "capture stress".

Total larynx weight was used to determine the response of the larynx in animals from experimental and control study sites. Adult males from each site were selected randomly for a total of 170 animals.

Testes were processed for routine paraffin embedding, serially sectioned at 5 μm, and stained with hematoxylin and eosin.

Three slides were selected: slide A was selected from the rostral terminus of the testes on the basis of the first observation of a seminiferous tubule; slide B was selected from the middle; and slide C was selected from a similar arbitrarily assigned caudal region of the testis with a complete seminiferous tubule. Three fields were photographed from each of the three slides yielding nine photomicrographs of each testis. The photomicrographs were overlain with a grid and the cell/tissue type under each crossbar was identified and counted by three different readers to determine the representative fractional volume of spermatogonia, spermatocyte, sperm, connective tissue, blood vessel "other" morphological features.

For oocytes, every 200th micron section was evaluated.

II. RESULTS and DISCUSSION: [All results discussed in this section and the next are those reported by the study authors. Although supplemental data are typically used in a qualitative manner only, EFED verified spreadsheet data and ran basic statistical analyses on the major study parameters. See attached appendix. If results differed in any substantive way, the difference was reported in the text below.]

The researchers were unable to collect Stage 66 metamorphs except in reference site C3; therefore, the study focused on adults. According to the report, the targeted number of fish, i.e., 20 males and 20 females per site, could not be achieved (**Table 1**) at sites E1 and E8 due to the introduction of sharptooth catfish; however, no explanation is provided as to why the targeted number could not be obtained from reference site C3.

Table 2 presents the mean body weight and snout-vent lengths (± standard deviation) of frogs collected in reference and experimental sites. There was no statistical difference in the average SVL of females collected from reference sites (71.8 ±4.45 mm) compared with females collected from experimental sites (73.9 ± 10.1 mm). Additionally, there was no difference in mean weight of females from reference sites (36.5 ± 4.32 g) and experimental sites (42.8 ± 15.7 g).

There was no statistical difference in the average SVL of males collected from reference sites (62.4 ± 8.45 mm) compared with males collected from experimental sites (62.6 ± 2.87 mm). There was also no statistical difference in mean weight of males collected at reference (25.8 ± 9.93 g) and experimental sites (27.0 ± 4.14 g).

While the report states that there was no difference in weight of the left testis from reference (18.6 mg) and experimental sites (23.3 mg) nor between the weight of the right testis from reference (15.8 mg) and experimental (22.1 mg) site collected animals, there were differences in the total testicular weight (combined right and left testis) between reference and experimental collection sites. The mean testes weight from control (0.037 ± 0.021 mg) was statistically different (ANOVA, $p < 0.0449$) than mean weight of testes (0.046 ± 0.018 mg) from frogs collected at experimental sites.

Table 2. Mean snout-vent length and body weight of male and female adult frogs collected at experimental (E1 - E8) and reference (R1 - R3) sites.

Pond	Snout-Vent Length (mm)		Body Weight (g)	
	Males	Females	Males	Females
E1	59.20 ± 9.49	64.48 ± 14.3	23.39 ± 14.4	29.15 ± 19.2
E3	66.63 ± 6.99	74.39 ± 6.94	32.57 ± 8.99	39.38 ± 8.47
E4	63.83 ± 3.35	77.54 ± 10.9	29.90 ± 4.17	48.82 ± 18.5

E6	62.66 ± 9.58	88.50 ± 9.53	26.11 ± 12.4	66.96 ± 22.7
E8	60.72 ± 7.28	64.47 ± 13.8	23.09 ± 8.79	29.80 ± 18.9
R1	58.00 ± 6.64	69.55 ± 8.02	19.44 ± 6.10	32.83 ± 11.1
R3	56.99 ± 10.9	68.91 ± 14.5	20.75 ± 13.3	35.35 ± 20.2
R8	72.11 ± 7.19	76.95 ± 7.04	37.26 ± 12.0	41.25 ± 10.4

Average condition indices (body weight ÷ snout-vent length) for females collected from reference sites (0.48 ± 0.15 g/mm) were not significantly different ($p > 0.0562$) from females collected at experimental sites (0.54 ± 0.20 g/mm). Similarly, male CI between reference (0.39 ± 0.14 g/mm) and experimental sites (0.42 ± 0.12 g/mm) were not statistically different ($p > 0.237$).

The report states that mean gonadalsomatic indices (GSI = total weight of gonad ÷ body weight) were lower for males collected in reference sites than in experimental sites but were not statistically different. EFED's analysis (see attached SAS output), though, indicates that GSI were significantly different (ANOVA, $p < 0.0001$) for males collected from reference (0.13 ± 0.04) sites compared to males collected from experimental sites (0.17 ± 0.04).

Morphological abnormalities (deformed limbs) were observed in 3 out of 212 (0.01%) of the animals and were attributed to attacks by the Cape terrapin (Pelomedusa subrufa).

Based on gross morphology, only 2 out of 101 males examined had discontinuous gonads, and 4 had testes of unequal size.

The extent of morphological changes in adult frog testes was determined by evaluating 56 testes from reference sites and 43 from experimental sites (**Table 3**). There were no statistical differences in the cell types comprising testes collected from reference and experimental sites.

Testicular oocytes were observed in 3% of the animals collected from reference sites and 2% of the frogs collected from experimental sites.

The study claims that laryngeal somatic index for males and females from reference and experimental sites were not significantly different ($p > 0.05$); however, no raw data were provided to verify these observations.

Table 3. Mean cell type identified in testes of male frogs collected from reference (C1, C3 and C6) and experimental (E1, E3, E4, E6 and E8) sites along with associated t-test p-values.

Site	Spermatogonia	Spermatocytes	Sperm	Connective Tissue and Other Cells	Blood Vessels
C1	3.0	25.6	55.9	14.9	0.5
C3	4.5	34.3	46.3	14.6	0.3
C6	2.8	22.6	60.3	13.9	0.3
E1	3.4	28.8	53.4	13.7	0.8
E3	3.6	24.9	58.1	13.2	0.2
E4	3.0	24.5	59.8	12.6	0.2
E6	3.2	20.8	62.4	13.2	0.3
E8	2.9	30.1	52.2	14.5	0.2
Mean of C1 - C6	3.5	27.5	54.2	14.5	0.4
Mean of E1 - E8	3.2	25.8	57.2	13.5	0.3
t-test p values	0.6214	0.6382	0.4782	0.0754	0.7305

C. REPORTED STATISTICS: Statistical analyses of frog numbers at each site were conducted using standard methods such as Chi-square, Kruskal-Wallis and Student's t-test. The initial analyses of histopathology were carried out using standard ANOVA procedures followed by Tukey's multi-comparison test using Minitab® statistical software.

D. VERIFICATION OF STATISTICAL RESULTS: Statistical analyses conducted using SAS (Statistical Analysis System, Release 8.01, Cary, North Carolina). See attached printout.

E. STUDY DEFICIENCIES:

Atrazine and/or triazine degradates were present in control sites.

While the low sample size at sites E1 and E8 may have been explained by the sudden introduction of predatory catfish, it doesn't explain why the remaining corn-growing sites (E3, E4 and E6) also yielded fewer frogs than non-corn growing sites.

Because frogs were sampled over disproportionate period of time, e.g., 6 months for site E8,animals were at different stages of development.

Frog traps were baited with liver/meat scraps that may have contained hormones.

Following capture, frogs were housed individually in "clean water" for 48 hours. The volume and characteristics of this water were not described in the study.

F. REVIEWER'S COMMENTS:

Atrazine is typically applied in October / November when corn is planted in South Africa. Frog sampling was conducted after the rainy season in April to May, and water samples to characterize exposure were collected within a time span of four weeks before frogs were sampled. At exposure site E8, frogs were sampled over a six-month time frame; the authors attributed the fewer number of frogs to a high runoff event that washed catfish into the pond and the catfish preyed on the frogs. Given the aerial photos and the fact that the majority of the ponds relied on rain (runoff) and were not dependent on a stream, it is unclear where the catfish came from. Because *Clarius* is an aquaculture species, it is possible that aquaculture ponds were in the vicinity of E1 and E8.

Frogs were attracted into baited traps containing liver and/or scrap meat that may have contained hormones. Apparently trapped animals were held in cages for two days which would likely have provided sufficient time to consume all of the bait. Also, depending on catch rates, the loading within the cages could have stressed the frogs. Although it doesn't state the desired sample size in the report's methodology, apparently (based on the raw data), 20 male and 20 female frogs were to be sampled at each site. **Table 1** shows that while the desired sample size was generally available for frogs collected in non-corn growing areas, sample sizes were minimally 50% lower in corn-growing areas. In site E1, sampling for female frogs extended into late June and at site E8 sampling extended into mid-August for females and into mid-September for males. Extended sampling periods at these two sites may have resulted in collecting animals that were at considerably different states of development. The gonadosomatic index and ovarian aromatase activity in females collected from sites E1 and E8 expressed the highest variability relative to any of the other treatment sites.

Table 1. Summary of total number of *Xenopus laevis* collected from April through May 2002 at non-corn growing (C) and corn growing (E) sites in South Africa.

Site	Males	Females
C1	20 (Apr 21 - Apr 23)	20 (Apr 21 - May 1)
C3	17 (Apr 4 - Apr 11)	20 (Apr 4 - Apr 11)

C6	20 (May 13 - May 15)	20 (May 13 - May 14)
E1	8 (April 29 - May 7)	8 (April 29 - June 29)
E3	10 (Apr 9 - Apr 17)	10 (Apr 9 - Apr 18)
E4	10 (Apr 13 - Apr 15)	10 (Apr 14 - Apr 16)
E6	10 (May 5 - May 9)	10 (May 5 - May 6)
E8	6 (Apr 29 - Sep 17)	15 (Apr 29 - Aug 17)

G. CONCLUSIONS:

This report is part of a multiphase study examining the effects of atrazine on African clawed frogs (*Xenopus laevis*) in their native habitat (South Africa). This phase of the study summarizes the body weights, lengths and condition index (weight/length) of both male and female collected from reference site (no corn grown and no atrazine/triazine use) and from experimental site (corn grown and atrazine/triazines used) ponds. The study also examined several aspects of gonadal growth (testis weight, gonadosomatic index) and development (incidence of deformities, cellular morphology) in frog testes collected from the two sites to determine whether atrazine exposure had affected these measurement endpoints.

While males were smaller than females at both study sites, there was no difference in weight or length of males or females collected at reference or experimental sites. Condition indices for males and females did not differ between reference and experimental collection sites. However, the mean testes weight from reference ponds (0.037 ± 0.021 mg) were statistically different than mean weight of testes (0.046 ± 0.018 mg) from frogs collected at experimental sites. GSI was significantly different for males collected from reference (0.13 ± 0.04) compared to males collected from experimental sites (0.17 ± 0.04).

There were no statistical differences in the cellular morphology of testes collected at reference sites and those collected at experimental site. The incidence of external abnormalities (missing limbs) was 1% across all animals collected (reference and experimental) and was attributed to the predatory effects of turtles. The incidence of testicular oocytes was 3% in males collected from reference ponds and 2% in experimental ponds. The study claims that laryngeal somatic index for males and females from reference and experimental sites were not significantly different ($p > 0.05$); however, no raw data were provided with which to verify these observations..

Although not discussed at length in this phase of the study, the interpretation of these data is complicated by the fact that atrazine, its degradates and other triazines were detected at significant levels in reference ponds. Additionally, at some sites, frogs were collected over a protracted period of time (6 months) rendering GSI a questionable metric. This study provides evidence that under the conditions studied, the incidence of testicular oocytes in *Xenopus laevis* in South Africa is roughly 2 to 3%. Because of the presence of atrazine in both sampling areas, a regression-based analysis would be more appropriate than hypothesis-testing based analysis. This study, though, was not designed for regression analysis. Can you include a summary conclusion statement here? Anything useful in this study? After the study is completed, a more definitive conclusion will be reached.

MEAN MALE SVL FOR REFERENCE (R) AND EXPERIMENTAL (E) COLLECTION SITES 109

Obs	POND	_TYPE_	_FREQ_	MEAN	STD
1	E1	0	8	59.2000	9.4870
2	E3	0	10	66.6300	6.9863
3	E4	0	10	63.8300	3.3549
4	E6	0	10	62.6600	9.5755
5	E8	0	6	60.7167	7.2841
6	R1	0	20	58.0000	6.6373
7	R3	0	17	56.9882	10.9170
8	R6	0	20	72.1100	7.1908

ANOVA FOR MALE SLV BETWEEN SITES 110

The ANOVA Procedure
Class Level Information

Class	Levels	Values
SITE	2	C E

Number of observations 101

Dependent Variable: LENGTH

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	1	2.016502	2.016502	0.02	0.8822
Error	99	9041.101320	91.324256		
Corrected Total	100	9043.117822			

R-Square	Coeff Var	Root MSE	LENGTH Mean
0.000223	15.22363	9.556373	62.77327

Source	DF	Anova SS	Mean Square	F Value	Pr > F
SITE	1	2.01650201	2.01650201	0.02	0.8822

Levene's Test for Homogeneity of LENGTH Variance
ANOVA of Squared Deviations from Group Means

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
SITE	1	77939.9	77939.9	6.28	0.0139
Error	99	1229054	12414.7		

Bartlett's Test for Homogeneity of LENGTH Variance

Source	DF	Chi-Square	Pr > ChiSq
SITE	1	5.1814	0.0228

Dunnett's t Tests for LENGTH

NOTE: This test controls the Type I experimentwise error for comparisons of all treatments against a control.

Alpha	0.05
Error Degrees of Freedom	99
Error Mean Square	91.32426
Critical Value of Dunnett's t	1.98422

Comparisons significant at the 0.05 level are indicated by ***.

SITE Comparison	Difference Between Means	Simultaneous 95% Confidence Limits	
E - C	0.2850	-3.5203	4.0902

NONPARAMETRIC COMPARISON OF MALE LENGTH BETWEEN SITES 114

The NPAR1WAY Procedure

Wilcoxon Scores (Rank Sums) for Variable LENGTH
Classified by Variable SITE

SITE	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
C	57	2872.50	2907.0	145.999621	50.394737
E	44	2278.50	2244.0	145.999621	51.784091

Average scores were used for ties.

Wilcoxon Two-Sample Test

Statistic	2278.5000
Normal Approximation	
Z	0.2329
One-Sided Pr > Z	0.4079
Two-Sided Pr > |Z|	0.8159
t Approximation	
One-Sided Pr > Z	0.4082
Two-Sided Pr > |Z|	0.8163

Z includes a continuity correction of 0.5.

Kruskal-Wallis Test

Chi-Square	0.0558
DF	1
Pr > Chi-Square	0.8132

Median Scores (Number of Points Above Median) for Variable LENGTH
Classified by Variable SITE

SITE	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
C	57	29.0	28.217822	2.503874	0.508772
E	44	21.0	21.782178	2.503874	0.477273

Average scores were used for ties.

Median Two-Sample Test

Statistic	21.0000
Z	-0.3124
One-Sided Pr < Z	0.3774
Two-Sided Pr > |Z|	0.7547

Median One-Way Analysis

Chi-Square	0.0976
DF	1
Pr > Chi-Square	0.7547

MEAN FEMALE SVL FOR REFERENCE (R) AND EXPERIMENTAL (E) COLLECTION SITES 116

Obs	POND	_TYPE_	_FREQ_	MEAN	STD
1	E1	0	6	64.4833	14.3272
2	E3	0	10	74.3900	6.9437
3	E4	0	10	77.5400	10.9218
4	E6	0	10	88.5000	9.5274
5	E8	0	15	64.4733	13.8195
6	R1	0	20	89.5450	91.3941
7	R3	0	20	68.9100	14.4936
8	R6	0	20	76.9500	7.0433

ANOVA FOR FEMALE SLV BETWEEN SITES

The ANOVA Procedure

Class Level Information

Class	Levels	Values
SITE	2	C E

Number of observations 111

Dependent Variable: LENGTH

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	1	628.8675	628.8675	0.38	0.5363
Error	109	178119.4467	1634.1234		
Corrected Total	110	178748.3142			

R-Square	Coeff Var	Root MSE	LENGTH Mean
0.003518	52.99887	40.42429	76.27387

Source	DF	Anova SS	Mean Square	F Value	Pr > F
SITE	1	628.8675382	628.8675382	0.38	0.5363

Levene's Test for Homogeneity of LENGTH Variance
ANOVA of Squared Deviations from Group Means

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
SITE	1	1.864E8	1.864E8	0.83	0.3656
Error	109	2.461E10	2.2581E8		

Bartlett's Test for Homogeneity of LENGTH Variance

Source	DF	Chi-Square	Pr > ChiSq
SITE	1	70.8565	<.0001

Dunnett's t Tests for LENGTH

NOTE: This test controls the Type I experimentwise error for comparisons of all treatments against a control.

Alpha	0.05
Error Degrees of Freedom	109
Error Mean Square	1634.123
Critical Value of Dunnett's t	1.98197

Comparisons significant at the 0.05 level are indicated by ***.

SITE Comparison	Difference Between Means	Simultaneous 95% Confidence Limits	
E - C	-4.776	-20.036	10.483

NONPARAMETRIC COMPARISON OF FEMALE LENGTH ACROSS SITES 121

The NPAR1WAY Procedure

Wilcoxon Scores (Rank Sums) for Variable LENGTH
Classified by Variable SITE

SITE	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
C	60	3256.0	3360.0	168.985548	54.266667
E	51	2960.0	2856.0	168.985548	58.039216

Average scores were used for ties.

Wilcoxon Two-Sample Test

Statistic	2960.0000
Normal Approximation	
Z	0.6125
One-Sided Pr > Z	0.2701
Two-Sided Pr > |Z|	0.5402
t Approximation	
One-Sided Pr > Z	0.2707
Two-Sided Pr > |Z|	0.5415

Z includes a continuity correction of 0.5.

Kruskal-Wallis Test

Chi-Square	0.3788
DF	1
Pr > Chi-Square	0.5383

Median Scores (Number of Points Above Median) for Variable LENGTH
Classified by Variable SITE

SITE	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
C	60	27.0	29.729730	2.637040	0.450000
E	51	28.0	25.270270	2.637040	0.549020

Average scores were used for ties.

Median Two-Sample Test

Statistic	28.0000
Z	1.0351
One-Sided Pr > Z	0.1503
Two-Sided Pr > |Z|	0.3006

Median One-Way Analysis

Chi-Square	1.0715
DF	1
Pr > Chi-Square	0.3006

AVERAGE LENGTH OF MALE AND FEMALE FROGS AT REFERENCE AND EXPERIMENTAL SITES 123

Obs	SITE	SEX	_TYPE_	_FREQ_	LENGTH	SD_L
1	C	F	0	3	78.4683	10.4010
2	C	M	0	3	62.3661	8.4536
3	E	F	0	5	73.8773	10.0521
4	E	M	0	5	62.6073	2.8664

MEAN MALE WEIGHT FOR REFERENCE (R) AND EXPERIMENTAL (E) COLLECTION SITES 124

Obs	POND	_TYPE_	_FREQ_	MEAN	STD
1	E1	0	8	23.3888	14.4006
2	E3	0	10	32.5740	8.9938
3	E4	0	10	29.9000	4.1673
4	E6	0	10	26.1050	12.3935
5	E8	0	6	23.0917	8.7925
6	R1	0	20	19.4445	6.1022
7	R3	0	17	20.7541	13.2996
8	R6	0	20	37.2590	12.0340

ANOVA FOR MALE WEIGHT BETWEEN SITES 125

The ANOVA Procedure

Class Level Information

Class	Levels	Values
SITE	2	C E

Number of observations 101

Dependent Variable: WEIGHT

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	1	52.00466	52.00466	0.35	0.5572
Error	99	14842.45191	149.92376		
Corrected Total	100	14894.45657			

R-Square	Coeff Var	Root MSE	WEIGHT Mean
0.003492	45.83106	12.24434	26.71624

Source	DF	Anova SS	Mean Square	F Value	Pr > F
SITE	1	52.00466491	52.00466491	0.35	0.5572

Levene's Test for Homogeneity of WEIGHT Variance
ANOVA of Squared Deviations from Group Means

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
SITE	1	126859	126859	2.23	0.1387
Error	99	5637162	56941.0		

Bartlett's Test for Homogeneity of WEIGHT Variance

Source	DF	Chi-Square	Pr > ChiSq
SITE	1	3.0051	0.0830

Dunnett's t Tests for WEIGHT

NOTE: This test controls the Type I experimentwise error for comparisons of all treatments against a control.

Alpha	0.05
Error Degrees of Freedom	99
Error Mean Square	149.9238
Critical Value of Dunnett's t	1.98422

Comparisons significant at the 0.05 level are indicated by ***.

SITE Comparison	Difference Between Means	Simultaneous 95% Confidence Limits	
E - C	1.447	-3.428	6.323

NONPARAMETRIC COMPARISON OF MALE WEIGHT BETWEEN SITES 129

The NPAR1WAY Procedure

Wilcoxon Scores (Rank Sums) for Variable WEIGHT
Classified by Variable SITE

SITE	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
C	57	2742.0	2907.0	146.005999	48.105263
E	44	2409.0	2244.0	146.005999	54.750000

Average scores were used for ties.

Wilcoxon Two-Sample Test

Statistic	2409.0000
Normal Approximation	
Z	1.1267
One-Sided Pr > Z	0.1299
Two-Sided Pr > |Z|	0.2599
t Approximation	
One-Sided Pr > Z	0.1313
Two-Sided Pr > |Z|	0.2626

Z includes a continuity correction of 0.5.

Kruskal-Wallis Test

Chi-Square	1.2771
DF	1
Pr > Chi-Square	0.2584

Median Scores (Number of Points Above Median) for Variable WEIGHT
Classified by Variable SITE

SITE	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
C	57	26.0	28.217822	2.503874	0.456140
E	44	24.0	21.782178	2.503874	0.545455

Average scores were used for ties.

Median Two-Sample Test

Statistic	24.0000
Z	0.8858
One-Sided Pr > Z	0.1879
Two-Sided Pr > |Z|	0.3757

Median One-Way Analysis

Chi-Square	0.7846
DF	1
Pr > Chi-Square	0.3757

MEAN FEMALE WEIGHT FOR REFERENCE (R) AND EXPERIMENTAL (E) COLLECTION SITES 131

Obs	POND	_TYPE_	_FREQ_	MEAN	STD
1	E1	0	6	29.1450	19.2315
2	E3	0	10	39.3810	8.4697
3	E4	0	10	48.8210	18.5235
4	E6	0	10	66.9560	22.6873
5	E8	0	15	29.8047	18.8819
6	R1	0	20	32.8315	11.1449
7	R3	0	20	35.3455	20.2334
8	R6	0	20	41.2495	10.4261

ANOVA FOR FEMALE WEIGHT BETWEEN SITES 132

The ANOVA Procedure

Class Level Information

Class	Levels	Values
SITE	2	C E

Number of observations 111

Dependent Variable: WEIGHT

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	1	1040.14605	1040.14605	2.97	0.0874
Error	109	38122.22029	349.74514		
Corrected Total	110	39162.36634			

R-Square	Coeff Var	Root MSE	WEIGHT Mean
0.026560	47.58918	18.70147	39.29775

Source	DF	Anova SS	Mean Square	F Value	Pr > F
SITE	1	1040.146048	1040.146048	2.97	0.0874

Levene's Test for Homogeneity of WEIGHT Variance
ANOVA of Squared Deviations from Group Means

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
SITE	1	2126150	2126150	5.83	0.0174
Error	109	39770440	364866		

Bartlett's Test for Homogeneity of WEIGHT Variance

Source	DF	Chi-Square	Pr > ChiSq
SITE	1	9.1873	0.0024

Dunnett's t Tests for WEIGHT

NOTE: This test controls the Type I experimentwise error for comparisons of all treatments against a control.

Alpha	0.05
Error Degrees of Freedom	109
Error Mean Square	349.7451
Critical Value of Dunnett's t	1.98197

Comparisons significant at the 0.05 level are indicated by ***.

SITE Comparison	Difference Between Means	Simultaneous 95% Confidence Limits	
E - C	6.143	-0.917	13.202

NONPARAMETRIC COMPARISON OF FEMALE WEIGHT ACROSS SITES 136

The NPAR1WAY Procedure

Wilcoxon Scores (Rank Sums) for Variable WEIGHT
Classified by Variable SITE

SITE	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
C	60	3132.50	3360.0	168.996671	52.208333
E	51	3083.50	2856.0	168.996671	60.460784

Average scores were used for ties.

Wilcoxon Two-Sample Test

Statistic	3083.5000
Normal Approximation	
Z	1.3432
One-Sided Pr > Z	0.0896
Two-Sided Pr > |Z|	0.1792
t Approximation	
One-Sided Pr > Z	0.0910
Two-Sided Pr > |Z|	0.1820

Z includes a continuity correction of 0.5.

Kruskal-Wallis Test

Chi-Square	1.8122
DF	1
Pr > Chi-Square	0.1782

Median Scores (Number of Points Above Median) for Variable WEIGHT
Classified by Variable SITE

SITE	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
C	60	27.0	29.729730	2.637040	0.450000
E	51	28.0	25.270270	2.637040	0.549020

Average scores were used for ties.

Median Two-Sample Test

Statistic	28.0000
Z	1.0351
One-Sided Pr > Z	0.1503
Two-Sided Pr > |Z|	0.3006

Median One-Way Analysis

Chi-Square	1.0715
DF	1
Pr > Chi-Square	0.3006

AVERAGE WEIGHT OF MALE AND FEMALE FROGS AT REFERENCE AND EXPERIMENTAL SITES 138

Obs	SITE	SEX	_TYPE_	_FREQ_	WEIGHT	SD_L
1	C	F	0	3	36.4755	4.3213
2	C	M	0	3	25.8192	9.9288
3	E	F	0	5	42.8215	15.7066
4	E	M	0	5	27.0119	4.1411

MEAN TOTAL WEIGHT OF TESTES (LEFT AND RIGHT COMBINED) BY POND 139

Obs	POND	_TYPE_	_FREQ_	WEIGHT	SD_W
1	E1	0	8	0.036925	0.014242
2	E3	0	10	0.053570	0.017665
3	E4	0	10	0.057670	0.009618
4	E6	0	10	0.033560	0.018074
5	E8	0	6	0.044200	0.019885
6	R1	0	29	0.022082	0.008848
7	R3	0	17	0.030953	0.023958
8	R6	0	30	0.051621	0.013727

MEAN TOTAL WEIGHT OF TESTES (LEFT AND RIGHT COMBINED) BY SITE 140

Obs	SITE	_TYPE_	_FREQ_	WEIGHT	SD_W
1	C	0	76	0.037232	0.021104
2	E	0	44	0.045650	0.018130

ANOVA FOR TOTAL TESTES WEIGHT BETWEEN REFERENCE AND EXPERIMENTAL SITES 141

The ANOVA Procedure

Class Level Information

Class	Levels	Values
SITE	2	C E

Number of observations 120

NOTE: Due to missing values, only 91 observations can be used in this analysis.

Dependent Variable: T_WT

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	1	0.00161041	0.00161041	4.14	0.0449
Error	89	0.03462027	0.00038899		
Corrected Total	90	0.03623068			

R-Square	Coeff Var	Root MSE	T_WT Mean
0.044449	47.75261	0.019723	0.041302

Source	DF	Anova SS	Mean Square	F Value	Pr > F
SITE	1	0.00161041	0.00161041	4.14	0.0449

Levene's Test for Homogeneity of T_WT Variance
ANOVA of Squared Deviations from Group Means

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
SITE	1	2.989E-7	2.989E-7	1.81	0.1823
Error	89	0.000015	1.654E-7		

Bartlett's Test for Homogeneity of T_WT Variance

Source	DF	Chi-Square	Pr > ChiSq
SITE	1	1.0070	0.3156

Dunnett's t Tests for T_WT

NOTE: This test controls the Type I experimentwise error for comparisons of all treatments against a control.

Alpha	0.05
Error Degrees of Freedom	89
Error Mean Square	0.000389
Critical Value of Dunnett's t	1.98699

Comparisons significant at the 0.05 level are indicated by ***.

SITE Comparison	Difference Between Means	Simultaneous 95% Confidence Limits		
E - C	0.008418	0.000197	0.016639	***

NONPARAMETRIC COMPARISON OF TOTAL TESTES WEIGHT BETWEEN REFERENCE AND EXPERIMENTAL SITES 145

The NPAR1WAY Procedure

Wilcoxon Scores (Rank Sums) for Variable T_WT
Classified by Variable SITE

SITE	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
C	47	1900.0	2162.0	125.912307	40.425532
E	44	2286.0	2024.0	125.912307	51.954545

Average scores were used for ties.

Wilcoxon Two-Sample Test

Statistic	2286.0000
Normal Approximation	
Z	2.0768
One-Sided Pr > Z	0.0189
Two-Sided Pr > |Z|	0.0378
t Approximation	
One-Sided Pr > Z	0.0203
Two-Sided Pr > |Z|	0.0407

Z includes a continuity correction of 0.5.

Kruskal-Wallis Test

Chi-Square	4.3298
DF	1
Pr > Chi-Square	0.0375

Median Scores (Number of Points Above Median) for Variable T_WT
Classified by Variable SITE

SITE	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
C	47	19.0	23.241758	2.396612	0.404255
E	44	26.0	21.758242	2.396612	0.590909

Average scores were used for ties.

Median Two-Sample Test

Statistic	26.0000
Z	1.7699
One-Sided Pr > Z	0.0384
Two-Sided Pr > |Z|	0.0767

Median One-Way Analysis

Chi-Square	3.1325
DF	1
Pr > Chi-Square	0.0767

MEAN FEMALE CONDITION INDEX BY CITE 147

Obs	SITE	_TYPE_	_FREQ_	CI	SD_CI
1	C	0	61	0.47898	0.15083
2	E	0	51	0.54278	0.19533

ANOVA FOR FEMALE CONDITION INDEX BETWEEN REFERENCE AND EXPERIMENTAL SITE 148

The ANOVA Procedure

Class Level Information

Class	Levels	Values
SITE	2	C E

Number of observations 112

NOTE: Due to missing values, only 110 observations can be used in this analysis.

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	1	0.11133162	0.11133162	3.73	0.0562
Error	108	3.22722541	0.02988172		
Corrected Total	109	3.33855703			

R-Square	Coeff Var	Root MSE	CI Mean
0.033347	33.99081	0.172863	0.508559

Source	DF	Anova SS	Mean Square	F Value	Pr > F
SITE	1	0.11133162	0.11133162	3.73	0.0562

Levene's Test for Homogeneity of CI Variance
ANOVA of Squared Deviations from Group Means

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
SITE	1	0.00619	0.00619	3.05	0.0835
Error	108	0.2191	0.00203		

Bartlett's Test for Homogeneity of CI Variance

Source	DF	Chi-Square	Pr > ChiSq
SITE	1	3.5615	0.0591

Dunnett's t Tests for CI

NOTE: This test controls the Type I experimentwise error for comparisons of all treatments against a control.

Alpha	0.05
Error Degrees of Freedom	108
Error Mean Square	0.029882
Critical Value of Dunnett's t	1.98218

Comparisons significant at the 0.05 level are indicated by ***.

SITE Comparison	Difference Between Means	Simultaneous 95% Confidence Limits	
E - C	0.06380	-0.00172	0.12931

NONPARAMETRIC COMPARISON OF FEMALE CONDITION FACTOR BETWEEN REFERENCE AND EXPERIMENTAL SIT 152

The NPAR1WAY Procedure

Wilcoxon Scores (Rank Sums) for Variable CI
Classified by Variable SITE

SITE	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
C	59	2965.0	3274.50	166.833000	50.254237
E	51	3140.0	2830.50	166.833000	61.568627

Wilcoxon Two-Sample Test

Statistic	3140.0000
Normal Approximation	
Z	1.8522
One-Sided Pr > Z	0.0320
Two-Sided Pr > |Z|	0.0640
t Approximation	
One-Sided Pr > Z	0.0334
Two-Sided Pr > |Z|	0.0667

Z includes a continuity correction of 0.5.

Kruskal-Wallis Test

Chi-Square	3.4416
DF	1
Pr > Chi-Square	0.0636

Median Scores (Number of Points Above Median) for Variable CI
Classified by Variable SITE

SITE	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
C	59	24.0	29.50	2.627047	0.406780
E	51	31.0	25.50	2.627047	0.607843

Median Two-Sample Test

Statistic	31.0000
Z	2.0936
One-Sided Pr > Z	0.0181
Two-Sided Pr > |Z|	0.0363

Median One-Way Analysis

Chi-Square	4.3832
DF	1
Pr > Chi-Square	0.0363

MEAN MALE CONDITION INDEX BY SITE 154

Obs	SITE	_TYPE_	_FREQ_	CI	SD_CI
1	C	0	57	0.39435	0.13640
2	E	0	44	0.42482	0.11530

ANOVA FOR MALE CONDITION INDEX BETWEEN REFERENCE AND EXPERIMENTAL SITE 155

The ANOVA Procedure

Class Level Information

Class	Levels	Values
SITE	2	C E

Number of observations 101

Dependent Variable: CI

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	1	0.02304412	0.02304412	1.41	0.2372
Error	99	1.61346081	0.01629758		
Corrected Total	100	1.63650493			

R-Square	Coeff Var	Root MSE	CI Mean
0.014081	31.31858	0.127662	0.407624

Source	DF	Anova SS	Mean Square	F Value	Pr > F
SITE	1	0.02304412	0.02304412	1.41	0.2372

Levene's Test for Homogeneity of CI Variance
ANOVA of Squared Deviations from Group Means

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
SITE	1	0.000694	0.000694	1.37	0.2444
Error	99	0.0501	0.000506		

Bartlett's Test for Homogeneity of CI Variance

Source	DF	Chi-Square	Pr > ChiSq
SITE	1	1.3333	0.2482

Dunnett's t Tests for CI

NOTE: This test controls the Type I experimentwise error for comparisons of all treatments against a control.

Alpha	0.05
Error Degrees of Freedom	99
Error Mean Square	0.016298
Critical Value of Dunnett's t	1.98422

Comparisons significant at the 0.05 level are indicated by ***.

SITE Comparison	Difference Between Means	Simultaneous 95% Confidence Limits	
E - C	0.03046	-0.02037	0.08130

NONPARAMETRIC COMPARISON OF MALE CONDITION INDEX BETWEEN REFERENCE AND EXPERIMENTAL SITES 159

The NPAR1WAY Procedure

Wilcoxon Scores (Rank Sums) for Variable CI
Classified by Variable SITE

SITE	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
C	57	2680.0	2907.0	146.006849	47.017544
E	44	2471.0	2244.0	146.006849	56.159091

Wilcoxon Two-Sample Test

Statistic	2471.0000
Normal Approximation	
Z	1.5513
One-Sided Pr > Z	0.0604
Two-Sided Pr > |Z|	0.1208
t Approximation	
One-Sided Pr > Z	0.0620
Two-Sided Pr > |Z|	0.1240

Z includes a continuity correction of 0.5.

Kruskal-Wallis Test

Chi-Square	2.4172
DF	1
Pr > Chi-Square	0.1200

Median Scores (Number of Points Above Median) for Variable CI
Classified by Variable SITE

SITE	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
C	57	23.0	28.217822	2.503874	0.403509
E	44	27.0	21.782178	2.503874	0.613636

Median Two-Sample Test

Statistic	27.0000
Z	2.0839
One-Sided Pr > Z	0.0186
Two-Sided Pr > |Z|	0.0372

Median One-Way Analysis

Chi-Square	4.3426
DF	1
Pr > Chi-Square	0.0372

MEAN MALE GONADOSOMATIC INDEX (GSI) BY SITE 161

Obs	SITE	_TYPE_	_FREQ_	GSI	SD_GSI
1	C	0	76	0.13027	0.037421
2	E	0	44	0.16847	0.042300

ANOVA FOR MALE GONADOSOMATIC (GSI) BETWEEN REFERENCE AND EXPERIMENTAL SITE 162

The ANOVA Procedure

Class Level Information

Class	Levels	Values
SITE	2	C E

Number of observations 120

NOTE: Due to missing values, only 91 observations can be used in this analysis.

Dependent Variable: GSI

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	1	0.03315187	0.03315187	20.87	<.0001
Error	89	0.14135759	0.00158829		
Corrected Total	90	0.17450946			

R-Square	Coeff Var	Root MSE	GSI Mean
0.189972	26.79357	0.039853	0.148742

Source	DF	Anova SS	Mean Square	F Value	Pr > F
SITE	1	0.03315187	0.03315187	20.87	<.0001

Levene's Test for Homogeneity of GSI Variance
ANOVA of Squared Deviations from Group Means

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
SITE	1	3.249E-6	3.249E-6	0.72	0.3994
Error	89	0.000403	4.531E-6		

Bartlett's Test for Homogeneity of GSI Variance

Source	DF	Chi-Square	Pr > ChiSq
SITE	1	0.6603	0.4164

Dunnett's t Tests for GSI

NOTE: This test controls the Type I experimentwise error for comparisons of all treatments against a control.

Alpha	0.05
Error Degrees of Freedom	89
Error Mean Square	0.001588
Critical Value of Dunnett's t	1.98699

Comparisons significant at the 0.05 level are indicated by ***.

SITE Comparison	Difference Between Means	Simultaneous 95% Confidence Limits		
E - C	0.038194	0.021583	0.054806	***

NONPARAMETRIC COMPARISON OF MALE GONADOSOMATIC INDEX BETWEEN REFERENCE AND EXPERIMENTAL SIT 166

The NPAR1WAY Procedure

Wilcoxon Scores (Rank Sums) for Variable GSI
Classified by Variable SITE

SITE	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
C	47	1645.0	2162.0	125.915315	35.000
E	44	2541.0	2024.0	125.915315	57.750

Wilcoxon Two-Sample Test

Statistic	2541.0000
Normal Approximation	
Z	4.1020
One-Sided Pr > Z	<.0001
Two-Sided Pr > |Z|	<.0001
t Approximation	
One-Sided Pr > Z	<.0001
Two-Sided Pr > |Z|	<.0001

Z includes a continuity correction of 0.5.

Kruskal-Wallis Test

Chi-Square	16.8587
DF	1
Pr > Chi-Square	<.0001

Median Scores (Number of Points Above Median) for Variable GSI
Classified by Variable SITE

SITE	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
C	47	14.0	23.241758	2.396612	0.297872
E	44	31.0	21.758242	2.396612	0.704545

Median Two-Sample Test

Statistic	31.0000
Z	3.8562
One-Sided Pr > Z	<.0001
Two-Sided Pr > |Z|	0.0001

Median One-Way Analysis

Chi-Square	14.8701
DF	1
Pr > Chi-Square	0.0001

MEAN CELL TYPES FOR EXPERIMENTAL SITES 168

Obs	POND	_TYPE_	_FREQ_	total	Gonia	Sperma	Sperm	other	BV_avg
1	E1	0	8	33.725	3.425	28.7625	53.35	13.675	0.75
2	E3	0	10	33.340	3.640	24.8800	58.06	13.220	0.22
3	E4	0	10	32.500	2.970	24.4900	59.82	12.580	0.16
4	E6	0	10	33.960	3.240	20.7900	62.41	13.240	0.31
5	E8	0	5	33.740	2.880	30.1400	52.24	14.540	0.16

MEAN CELL TYPES FOR REFERENCE SITES 169

Obs	POND	_TYPE_	_FREQ_	total	Gonia	Sperma	Sperm	other	BV_avg
1	C1	0	19	34.9158	3.02105	25.5737	55.9316	14.9474	0.50526
2	C3	0	17	34.3941	4.53529	34.2882	46.2647	14.6353	0.28824
3	C6	0	20	34.3800	2.82000	22.6050	60.3200	13.9050	0.33500

AVERAGE CELL TYPES BY SITE 170

Obs	SITE	_TYPE_	_FREQ_	total	Gonia	Sperma	Sperm	other	BV_avg
1	C	0	3	34.5633	3.45878	27.4890	54.1721	14.4959	0.37617
2	E	0	5	33.4530	3.23100	25.8125	57.1760	13.4510	0.32000

Data Evaluation Report on Exposure of *Xenopus laevis* larvae to Different Concentrations of Atrazine in Semi-Natural Microcosms

EPA MRID Number 458677-11

Data Requirement:

EPA DP Barcode D288775

EPA MRID 458677-11
EPA Guideline 70-1(Special Study)

Test material: **Purity:** not reported
Common name: Atrazine
Chemical name: IUPAC
CAS name 6-chloro-N-ethyl-N'-(1-methylethyl)-1,3,5-triazine-2,4-diamine
CAS No. 1912-24-9
Synonyms
EPA PC Code: 80803

Thomas M Steeger

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EPA PC Code 080803

Date Evaluation Completed: 06/01/2003

CITATION: Du Preez, L., K. R. Solomon and A. M. 2003. Exposure of *Xenopus laevis* larvae to different concentrations of atrazine in semi-natural microcosms. School of Environmental Sciences and

Development, Zoology Department, Potchefstroom University of CHE, Potchefstroom 2520, South Africa. Sponsor: Syngenta Crop Protection, Inc., Laboratory Study ID ECORISK Number SA-01-D.

EXECUTIVE SUMMARY:

This is an interim report to assess the effects of atrazine on gonadal abnormalities in *Xenopus laevis* metamorphs through the use of microcosm studies. *X. laevis* adults were collected from an earthen pond north (pond C6) of Potchefstroom, South Africa, based on the reported absence of atrazine use and corn production in its watershed and the presence of *X. laevis*. Adults were induced to spawn in the laboratory and the 96-hr old progeny were divided among 4 treatments (0, 1, 10 and 25 µg/L atrazine) with three replicates per treatment (800 tadpoles per replicate). Microcosms consisted of 1600-L cement tanks, lined with polyethylene with a 3-cm sand bottom, and filled with 1,100 L of tap water. Larvae were exposed until they reached NF stage 66 (forelimb emergence and complete tail resorption); the study was terminated after 133 days of exposure. A total of 150 stage-66 frogs were sampled per treatment. Snout-vent length, weight, days to stage 66 (metamorphosis) were recorded and animals were examined for gross gonadal morphology.

Mean measured concentrations of atrazine ranged from 0.9 to 1.82, 10 to 15.9 and 23.8 to 39.7 µg/L for the 1, 10 and 25 µg/L nominal treatments, respectively. Atrazine in reference tanks ranged from 0 to 0.1 µg/L. Although some animals reached stage 66 by 70 days, the majority did not reach metamorphosis until between 126 to 133 days. The study authors attributed low water temperatures to the delayed development of the tadpoles. Based on gross morphology, the incidence of gonadal deformities in 1, 10 and 25 µg/L atrazine groups was 1.3, 0.7 and 3.3% of the total frogs examined (150), respectively; reference frogs exhibited a 4% incidence of gonadal deformities. Discontinuous testis was the only gonadal abnormality identified; no abnormalities were observed in the ovaries except for one ovary that was reduced in size. Males comprised 48%, 39% and 47% of the 1.0, 10 and 25 µg/L atrazine-treated samples, respectively, while the reference samples was 45% male.

This interim microcosm study represents a reasonable step forward in testing for effects observed in the laboratory and may provide useful information when all the analyses are completed. The present study, though, showed that developmental rates across all treatments, in terms of time to metamorphosis, were delayed. Fluctuating water quality in the microcosm units may have impacted the developmental rate. Additionally, the study was designed with the assumption that phytoplankton growth would serve as a source of food for developing tadpoles. Although the authors note that phytoplankton "flourished," no measure (*e.g.*, chlorophyl *a* concentration) of phytoplankton growth was reported and supplemental food (rabbit pellets) had to be provided to the developing tadpoles. However, phytoplankton growth could have been significantly limited by atrazine treatment, thereby resulting in indirect effects of atrazine on development. In support of this possibility, atrazine-treated male and female frogs were significantly smaller in terms of length and weight than their "untreated" counterparts, further complicating interpretation of the study results. This assessment was based on gross morphology alone, and the results may change when histology is completed.

I. MATERIALS AND METHODS

GUIDELINE FOLLOWED: Nonguideline Study

COMPLIANCE: Not conducted in accordance with the Good Laboratory Practices as outlined in 40 CFR Part 160, August 19, 1989. However, GLP elements were incorporated into study conduct including:

- This study was conducted in accordance with a written protocol, signed by the Study Director and Principal Investigator
- Standard Operating Procedures were developed/available for official activities.
- Protocol amendments/deviations were written and signed by the Study Director.

A. MATERIALS:

1. Test Material Atrazine

Description: Not reported

Lot No./Batch No. : Not reported

Purity: Not reported

Stability of compound uder Test Conditions: Not reported

Storage conditions of test chemicals:_ Not reported

2. Test organism:

Species: African clawed frog (*Xenopus laevis*)

Age at test initiation : 96-hr larvae

Weight at study initiation (mean and range): Not reported

Length at study initiation (mean and range): Not reported

Source: Adult *X. laevis* obtained from earthen pond (C6) 16 km north of the city of Potchefstroom, South Africa.

B. STUDY DESIGN:

Objective: 1. To assess the effects of exposure to atrazine on gonadal abnormalities in *Xenopus laevis* metamorphs and sub-adults under semi-natural conditions in microcosms.

1. Experimental Conditions

a) Range-finding Study:

b) Definitive Study

Table 1 . Experimental Parameters

Parameter	Details
Acclimation: period: Conditions: (same as test or not) Feeding: Health: (any mortality observed)	 Microcosms stabilized for 5 months Adults paired and injected with chorionic gonadotropin (Pregnyl®) to induce spawning and placed in 300 x 240 x 240 mm) spawning tanks at 30°C. After spawning, adults were removed from tanks and water was oxygenated.
Duration of the test	NF Stage 66 (forelimb emergence and tail resorbtion) or 19 weeks total exposure whichever came first.
Test condition static/flow- through Type of dilution system for flow-through method. Renewal rate for static renewal	 static water maintained at 1,100 L mark by addition of tap water when necessary
Aeration, if any	not reported
Test vessel Material: (glass/stainless steel) Size: Fill volume:	 Cement with polyethylene liner 2.25 m x 0.6 m x 1.2 m = 1.62 m^3 (1,620 L) 1,100 L plastic liner covered with 3-cm layer of sandy soil
Source of dilution water Quality:	Tap water

Parameter	Details
<u>Water parameters</u>: Hardness pH Dissolved oxygen Total Organic carbon Particulate Matter Ammonia Nitrite Metals Pesticides Chlorine Temperature {Salinity for marine or estuarine species} Intervals of water quality measurement	Measured every Thursday mid-morning.
Number of replicates/groups: negative control: water treated ones:	3
Number of organisms per replicate /groups:	800 (from two separate spawns: 600 from on Sept. 5th and 200 from Sept. 12th)
Biomass loading rate	800 tadpoles per 1,100 L
Test concentrations: nominal: measured:	 1, 10 and 25 µg/L 0.91 - 1.82 µg/L; 10 - 15.9 µg/L; 23.8 - 39.7 µg/L
Solvent (type, percentage, if used)	none
Lighting	natural sunlight
Feeding	supplemental rabbit food (Complete Rabbit Pellets); 20 grams homogenized in 500 ml of tap water provided once per week but later twice per week.

Parameter	Details
Recovery of chemical Level of Quantitation Level of Detection	GC/MS used to measure atrazine 0.1 µg/L
Positive control {if used, indicate the chemical and concentrations}	None
Other parameters, if any	

2. Observations:

Table 2: Observations

Criteria	Details
Parameters measured including the sublethal effects/toxicity symptoms	body weight, snout-vent length, time (days) to metamorphosis; gross morphology and histology of gonads.
Observation intervals	Every 14 days 1-L water samples collected for atrazine measurement; water chemistry weekly. From 11/5/02 until mid-January 2003, metamorphs removed from microcosms every 2nd day.
Were raw data included?	Yes
Other observations, if any	

Report notes that 800 tadpoles per microcosm were well below the effective density of 1 tadpole/L for a frog to reach metamorphosis successfully (Weldon 1999). *Xenopus* bucket traps baited with marrow bones used to catch metamorphs.

The report notes that 150 metamorphs were collected from each treatment. The researchers attempted to collect 50 from each of the three replicates; however, they were apparently unable to do this in all cases and therefore had to sample more from one replicate than the others. According to the protocol, no more than 75 metamorphs would be collected from a single tank.

II. RESULTS and DISCUSSION: [All results discussed in this section and the next are those reported by the study authors. Although supplemental data are typically used in a qualitative manner only, EFED verified spreadsheet data and ran basic statistical analyses on the major study parameters. See attached appendix. If results differed in any substantive way, the difference was reported in the text below.]
Atrazine concentrations were relatively consistent with nominal levels. On one occasion, atrazine was detected in one of the reference microcosms at a concentration equal to the method detection limit (0.1 µg/L).

Microcosms were colder than natural water bodies (no data provided). Extensive early mortality was observed in microcosm 1 (1 µg/L), while extensive mortality and slow development was observed in microcosm 8(10 µg/L); both were excluded from the analysis.

First metamorphs reached development stage 66 in the 10th week. Collection of metamorphs terminated in week 19 when the target number (150/treatment) was reached. The majority of frogs reaching stage 66 appears to have occurred between 126 and 133 days of exposure.

All 600 specimens (150 frogs/treatment * 4 treatments) were examined visually and under a dissecting microscope for gonadal deformities. Of the total number of frogs exposed to 1, 10 and 25 µg/L of atrazine, 1.33%, 0.67% and 3.33% exhibited gonadal deformities, respectively, compared to 4% in reference microcosms. No intersex gonads were observed. Discontinuous testis was the most frequently observed anomaly and ranged from 1.7% to 7.1% in atrazine -treated animals and 8.8% in controls. No gonadal abnormalities were detected in females except for one female with a reduced-size ovary. The study concludes that based on gross morphology, atrazine had no adverse effect on gonadal development at mean concentrations ranging from 1.4 to 30.8 µg/L under semi-field conditions.

Table 3. Total number of frogs examined and percentage of gonadal deformities.

Nominal Concentration (measured) µg/L	Total Number				Percent Gonadal Abnormalities	
	Frogs Collected	Males	Females	Gonadal Abnormalities	Total Frogs	Males
0 (0 - 0.1)	150	68	82	6	4.0	8.8
1 (0.91 - 1.82)	150	72	78	2	1.3	2.8
10 (10 - 15.9)	150	59	91	1	0.7	1.7
25 (23.8 - 39.7)	150	70	80	5	3.3	7.1

C. **REPORTED STATISTICS**: Little to no statistics are reported in this interim report.

D. **VERIFICATION OF STATISTICAL RESULTS**: Statistical analyses run using SAS® (Statistical Analysis System, Release 8.01, Cary, North Carolina); see attached output.

E. **STUDY DEFICIENCIES**: The purity of atrazine used to make up exposure solutions was not reported. The length of exposure for test animals differed; one group consisting of 600 were exposed for approximately one week longer than the complement of 200 additional animals added later. Tap water was used as the source of dilution water. Dissolved oxygen in several of the microcosms dropped below 3 mg/L. Exposure tanks were not all at the same temperature, but were dependent on the amount of shade from a nearby tree. The number of animals sampled from each replicate may have been different; a total of 150 were sampled from each treatment, but up to 75 may have been sampled from a replicate rather than 50 from each replicate. Atrazine was detected at 0.1 µg/L (detection limit of method) in a reference tank (microcosm 3)

F. REVIEWER'S COMMENTS:

Frogs were collected from site C6 because of the absence of atrazine use in the watershed; however, this site was characterized in previous studies (Smith *et al.* 2003) as having measurable atrazine concentrations averaging 0.15 µg/L. Although this concentration is relatively low, it does represent a level that has been reported to cause gonadal deformities in males (Hayes *et al.* 2002). The potential for gonadal deformities in the control limits the usefulness of this study in testing the hypothesis..

The conditions under which the tests were conducted probably resulted in poor water quality, which contributed to the relatively retarded development, although low temperatures in the initial few weeks of the study were also a factor. However, the exposure media was never changed and the total volume was not replenished to make up for evaporative losses. This approach would lead to the accumulation of waste products, especially since the study was run for 19 weeks. Unfortunately, mortality was not evaluated throughout the study, so it is difficult to assess the appropriateness of the exposure protocol. All that one can tell is that a maximum of 75 organisms were sampled from any one tank, representing less than 10% of the initial set. It is unclear why the study started out with so many organisms in each tank.

Microcosms 1 and 8 reportedly exhibited extensive mortality early in the study and the authors alluded to possible temperature shock because microcosm 1 was shaded; however, several of the microcosms were shaded by trees and it is unclear why only one would have had such excessively low temperature. Also, temperature shock usually results from too high a temperature, not too low a temperature. Microcosm 8 exhibited slow metamorphosis and the frogs were not at stage 66 by 133 days. Although microcosms were excluded from the stud, the cause of mortality and delayed development was not adequately characterized.

The method detection limit (0.1 µg/L) seems unusually high for GC/MS given that ELISA assays have a detection limit of 0.025 µg/L.

Frogs were relatively slow to reach metamorphosis stage 66. According to the authors, under controlled laboratory conditions of 20 - 25°C, *X. laevis* tadpoles take 58 days to complete metamorphosis (Nieuwkoop and Faber 1967) and under natural conditions where water temperatures may fluctuate widely, metamorphosis may take from 56 to 63 days, still a shorter period of time than the minimal 70 days required in the current study.

Because the test animals were added in two stages, exposure times differed. Animals (600) added to the tank first were exposed for roughly a week longer than the second complement (200) of animals added to bring the total number of tadpoles per microcosm to 800.

Although the first metamorphs reached stage 66 by 70 days, the majority of study animals did not reach stage 66 until 126 to 133 days, which implies that the study conditions delayed development and may have rendered the animals less sensitive to any developmental effects of atrazine should they exist.

Although raw water quality data were not provided, a series of figures depicted water temperature, pH, dissolved oxygen (DO) and conductivity over the study period. It appears from these graphs that something (perhaps a high rain event) happened around Day 90 to 100 that caused conductivity, temperature and pH to drop substantially. Conductivity in all but three of the exposure tanks tended to steadily increase from days 0 to 90; however, tanks 2,4, 6 and 9 had very erratic conductivity with steep declines. In tanks 4 and 6, conductivity plummeted Day 40 and in tank 2 conductivity plummeted by day 60.

By Day 100, dissolved oxygen in some replicates of the reference, 10 and 25 µg/L treatments dropped below 2 mg/L. Dissolved oxygen and conductivity in the 25 µg/L treatment were unusually variable relative to the other treatments. It is possible that the supplemental feeding resulted in food residues that increased conductivity during days 0 through 90 and that a high rain event may have resulted in a rapid influx of water that lowered conductivity and dissolved oxygen. The low oxygen content of the water is difficult to explain because water samples were collected mid-day when photosynthesis by phytoplankton should supplement dissolved oxygen levels. Heavy rain charged with carbon dioxide could have lowered DO; however, it should have also altered pH which does not appear to have occurred. However, given that the report states that very little rain fell during the study, it is uncertain why water quality changed so dramatically.

The study was intentionally designed to keep the loading rate below 1 tadpole per liter. Because the reference was from 1999, it suggests that the authors were aware that higher loading rates would have compromised the development of tadpoles.

Total time to metamorphosis cannot be determined from this study because an unknown number of animals were sacrificed by Day 133. It is likely that tadpoles could have died and not been accounted for during the course of the study. What is apparent, though, is that metamorphosis was considerably delayed for the majority of animals in this study.

Although no intersex gonads were observed, the current analysis is based on gross morphology alone and does not represent an analysis based on histology.

Although the stated objectives were to analyze snout-vent length (SVL) and mass, the report did not present an analysis of these data. In the table below, the mean SVL and mean mass for each treatment replicate were extracted from the appendix. No statistical analysis was done on this data set, but it appears as if there may be significant reductions in SVL and mass associated with atrazine exposure. EFED's analysis (see attached output) revealed that on average, male and female frogs not exposed to atrazine were longer 21.17 mm and 22.07 mm, respectively, than frogs from atrazine-treated groups (**Table 4**); non-exposed males and females also tended to weigh more, i.e., 1.08 and 1.20 g, respectively, than frogs treated with atrazine. Analysis of variance (see attached printout) revealed that both males and females were significantly larger in terms of weight ($p < 0.02$) and length ($p < 0.03$) than atrazine treated males and females. It is unclear whether the difference in size is due to the direct effects of atrazine on growth or whether it is due to reduced phytoplankton caused by atrazine treatment. At each treatment though, there was no difference in weight or length of males versus females.

Table 4. Summary of average male and female *Xenopus laevis* lengths and weights with associated standard deviations by atrazine concentration.

Atrazine Concentratio n	Sex	Length	Standard Deviation	Weight	Standard Deviation
0	Female	21.17	3.80	1.08	0.75
0	Male	22.07	3.97	1.20	0.78
1	Female	18.55	2.70	0.70	0.32
1	Male	18.78	2.36	0.71	0.28
10	Female	18.82	3.36	0.73	0.51
10	Male	18.84	3.17	0.75	0.61
25	Female	17.50	3.11	0.59	0.40
25	Male	18.17	2.79	0.64	0.34

Similarly, there was no analysis on time to metamorphosis, as originally stated in the objective section of the study. However, given the method by which metamorphs were removed, and given the confounding effects of the differential exposure during the first week of the study, this analysis may not be possible. Individual treatment data for each tank is needed for Figure 2. Clearly, the data were collected for each tank, but for some reason they were not presented in this study. Also, some very large organisms were collected that were up to 4 to 6 times the mean weight of the entire sample, suggesting that these organisms were not collected at completion of metamorphosis, but some time much later.

In terms of the gonadal analysis, only the gonads of the first 150 stage 66 organisms in each treatment were evaluated. This sampling would bias the evaluation toward the faster developing organisms, which is not representative of the population being tested. Only 150 organisms were sampled, representing 6.25% of the original organisms in a treatment.

H. REFERENCES:

Hayes, T. B., A. Collins, M. Lee, M. Mendoza, N. Noriega, A. S. Stuart, and A. Vonk. 2002*a*. Hermaphroditic, demasculinized frogs after exposure to the herbicide atrazine at low ecologically relevant doses. Proceedings of the National Academy of Sciences of the United States of America 99(8): 5476 - 5480

Nieuwkoop, P. D. and J. Faber. 1967. Normal table of *Xenopus laevis* (Daudin). North-Holland Publishing Company, Amsterdam.

Smith, E. E., L. Du Preez, and K. Solomon. 2003. Gonadal and laryngeal responses to field exposure of *Xenopus laevis* to atrazine in areas of corn production in South Africa. The Institute of Environmental and Human Health, Texas Tech University, Lubbock, TX and School of Environmental Sciences and Development, Potchefstroom University for CHE, Potchefstroom, South Africa. Sponsor: Syngenta Crop Protection, Inc., Laboratory Study ID: ECORISK Number SA-01C.

Weldon, C. 1999. The sustainable utilization of the African clawed frog *Xenopus laevis* (Daudin). M.Sc. Thesis. Department of Zoology and Entomology, University of the Orange Free State, Bloemfontein, South Africa.

MEAN LENGTH AND WEIGHT FOR ALL FROGS COLLECTED AT EACH SITE 621

Obs	ATRAZINE	SITE	_TYPE_	_FREQ_	LENGTH	WEIGHT	L_SD	W_SD
1	0	12	0	61	21.3213	1.09951	4.09761	0.76949
2	0	3	0	67	21.5955	1.10149	3.49729	0.67835
3	0	7	0	22	22.2409	1.34364	4.50816	0.96341
4	1	11	0	75	17.5907	0.57760	1.82380	0.18347
5	1	4	0	75	19.7347	0.83400	2.70368	0.34261
6	10	10	0	75	18.1227	0.61533	1.63381	0.20565
7	10	5	0	75	19.5400	0.86227	4.22665	0.73003
8	25	2	0	75	18.4040	0.66387	2.59393	0.37961
9	25	6	0	47	17.5574	0.60936	3.52807	0.39261
10	25	9	0	28	16.5679	0.48536	2.52866	0.28908

MEAN LENGTH AND WEIGHT FOR MALE AND FEMALE FROGS COLLECTED AT EACH SITE 622

Obs	ATRAZINE	SITE	Sex	_TYPE_	_FREQ_	LENGTH	WEIGHT	L_SD	W_SD
1	0	12	F	0	29	20.5931	1.03276	4.17312	0.81263
2	0	12	M	0	32	21.9813	1.16000	3.97772	0.73595
3	0	3	F	0	41	21.5585	1.07951	3.24700	0.59480
4	0	3	M	0	26	21.6538	1.13615	3.92624	0.80419
5	0	7	F	0	12	21.2417	1.19250	4.70483	1.07726
6	0	7	M	0	10	23.4400	1.52500	4.17591	0.82481
7	1	11	F	0	39	17.4103	0.56821	1.99272	0.18953
8	1	11	M	0	36	17.7861	0.58778	1.62659	0.17879
9	1	4	F	0	39	19.6974	0.84026	2.85671	0.36757
10	1	4	M	0	36	19.7750	0.82722	2.56742	0.31844
11	10	10	F	0	47	18.0426	0.61362	1.66417	0.21999
12	10	10	M	0	28	18.2571	0.61821	1.60241	0.18286
13	10	5	F	0	43	19.6791	0.86488	4.40317	0.68274
14	10	5	M	0	32	19.3531	0.85875	4.03908	0.80039
15	25	2	F	0	41	18.4561	0.68610	2.88037	0.44866
16	25	2	M	0	34	18.3412	0.63706	2.24150	0.27891
17	25	6	F	0	21	17.1619	0.57143	3.59256	0.39082
18	25	6	M	0	26	17.8769	0.64000	3.51287	0.39905
19	25	9	F	0	19	15.8316	0.41368	2.22862	0.18860
20	25	9	M	0	9	18.1222	0.63667	2.52971	0.40503

MEAN LENGTH AND WEIGHT FOR MALE AND FEMALE FROGS BY ATRAZINE CONC 623

Obs	ATRAZINE	Sex	_TYPE_	_FREQ_	LENGTH	WEIGHT	L_SD	W_SD
1	0	F	0	82	21.1707	1.07951	3.79852	0.75050
2	0	M	0	68	22.0706	1.20456	3.97068	0.77549
3	1	F	0	78	18.5538	0.70423	2.70405	0.32116
4	1	M	0	72	18.7806	0.70750	2.35723	0.28334
5	10	F	0	90	18.8244	0.73367	3.35507	0.51080
6	10	M	0	60	18.8417	0.74650	3.17032	0.60543
7	25	F	0	81	17.5049	0.59247	3.10676	0.39879
8	25	M	0	69	18.1377	0.63812	2.78832	0.34000

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            PLOT OF FROG WEIGHT OVER ATRAZINE CONCENTRATION FOR MALE AND FEMALE FROGS                624

-------------------------------------------- Sex=F --------------------------------------------

                                Plot of WEIGHT*ATRAZINE.  Symbol used is '*'.

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             0  1  2  3  4  5  6  7  8  9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25

                                              ATRAZINE
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             PLOT OF FROG WEIGHT OVER ATRAZINE CONCENTRATION FOR MALE AND FEMALE FROGS            625

-------------------------------------------- Sex=M --------------------------------------------

                                   Plot of WEIGHT*ATRAZINE.  Symbol used is '*'.

    1.2 ê  *
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           0  1  2  3  4  5  6  7  8  9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25

                                                 ATRAZINE
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ANALYSIS OF VARIANCE FOR FROG WEIGHT BETWEEN TREATMENT GROUPS BY SEX 626

-- Sex=F --

The GLM Procedure

Class Level Information

Class	Levels	Values
ATRAZINE	4	0 1 10 25

Number of observations 331

Dependent Variable: MASS

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	3	10.8440965	3.6146988	13.21	<.0001
Error	327	89.5108805	0.2737336		
Corrected Total	330	100.3549770			

R-Square	Coeff Var	Root MSE	MASS Mean
0.108057	67.26132	0.523196	0.777855

Source	DF	Type I SS	Mean Square	F Value	Pr > F
ATRAZINE	3	10.84409653	3.61469884	13.21	<.0001

Source	DF	Type III SS	Mean Square	F Value	Pr > F
ATRAZINE	3	10.84409653	3.61469884	13.21	<.0001

--- Sex=M ---

The GLM Procedure

Class Level Information

Class	Levels	Values
ATRAZINE	4	0 1 10 25

Number of observations 269

Dependent Variable: MASS

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	3	13.56982643	4.52327548	15.88	<.0001
Error	265	75.47985684	0.28482965		
Corrected Total	268	89.04968327			

R-Square	Coeff Var	Root MSE	MASS Mean
0.152385	64.76464	0.533694	0.824052

Source	DF	Type I SS	Mean Square	F Value	Pr > F
ATRAZINE	3	13.56982643	4.52327548	15.88	<.0001

Source	DF	Type III SS	Mean Square	F Value	Pr > F
ATRAZINE	3	13.56982643	4.52327548	15.88	<.0001

PROC UNIVARIATE OUTPUT FOR RESIDUALS FROM GLM PROCEDURE USING FROG WEIGHT BY SEX 630

-- Sex=F --

The UNIVARIATE Procedure
Variable: Resid

Moments

N	331	Sum Weights	331
Mean	0	Sum Observations	0
Std Deviation	0.52081195	Variance	0.27124509
Skewness	3.47817499	Kurtosis	16.8585986
Uncorrected SS	89.5108805	Corrected SS	89.5108805
Coeff Variation	.	Std Error Mean	0.02862641

Basic Statistical Measures

Location		Variability	
Mean	0.00000	Std Deviation	0.52081
Median	-0.12423	Variance	0.27125
Mode	-0.24951	Range	4.39000
		Interquartile Range	0.29944

Tests for Location: Mu0=0

Test	-Statistic-		-----p Value------	
Student's t	t	0	Pr > |t|	1.0000
Sign	M	-60.5	Pr >= |M|	<.0001
Signed Rank	S	-9276	Pr >= |S|	<.0001

Tests for Normality

Test	--Statistic---		-----p Value------	
Shapiro-Wilk	W	0.670492	Pr < W	<0.0001
Kolmogorov-Smirnov	D	0.218809	Pr > D	<0.0100
Cramer-von Mises	W-Sq	5.537051	Pr > W-Sq	<0.0050
Anderson-Darling	A-Sq	29.21036	Pr > A-Sq	<0.0050

Quantiles (Definition 5)

Quantile	Estimate
100% Max	3.6004878
99%	2.4504878
95%	0.9657692
90%	0.4175309
75% Q3	0.0557692
50% Median	-0.1242308
25% Q1	-0.2436667
10%	-0.3495122
5%	-0.4436667
1%	-0.7195122
0% Min	-0.7895122

Extreme Observations

------Lowest------		-----Highest-----	
Value	Obs	Value	Obs
-0.789512	1	2.18753	279
-0.739512	38	2.45049	31

-0.739512	6	2.92633	250
-0.719512	42	3.38049	71
-0.669512	7	3.60049	13

```
PROC UNIVARIATE OUTPUT FOR RESIDUALS FROM GLM PROCEDURE USING FROG WEIGHT BY SEX      632

------------------------------------------ Sex=F ------------------------------------------

                          The UNIVARIATE Procedure
                             Variable:  Resid

                  Histogram                              #              Boxplot
    3.7+*                                                1                 *
       .
       .*                                                1                 *
       .
       .*                                                1                 *
       .
       .*                                                1                 *
       .
       .*                                                1                 *
       .
       .*                                                1                 *
    1.5+*                                                3                 *
       .*                                                2                 *
       .**                                               4                 *
       .***                                              9                 0
       .**                                               4                 0
       .***                                              7                 0
       .******                                          18                 |
       .*****************                               52              +--+--+
       .**************************************         114              *-----*
       .*******************************                 92              +-----+
       .*****                                           13                 |
   -0.7+***                                              7                 0
        ----+----+----+----+----+----+----+---
        * may represent up to 3 counts


                          The UNIVARIATE Procedure
                             Variable:  Resid

                           Normal Probability Plot
    3.7+                                                    *
       |                                                    *
       |
       |                                                   *
       |                                                  *
       |                                                 *
       |
       |                                                *
       |                                               ***
    1.5+                                               *    ++
       |                                             ** ++++
       |                                           ****++
       |                                        ++*+
       |                                    ++++ **
       |                                ++++  ***
       |                            ++++ ******
       |                         **********
       |               ************
       |          *****    ++++
   -0.7+* ***       ++++
        +----+----+----+----+----+----+----+----+----+----+
             -2        -1         0        +1        +2
```

PROC UNIVARIATE OUTPUT FOR RESIDUALS FROM GLM PROCEDURE USING FROG WEIGHT BY SEX 634

-- Sex=M --

The UNIVARIATE Procedure
Variable: Resid

Moments

N	269	Sum Weights	269
Mean	0	Sum Observations	0
Std Deviation	0.53069884	Variance	0.28164126
Skewness	3.56627975	Kurtosis	19.3195663
Uncorrected SS	75.4798568	Corrected SS	75.4798568
Coeff Variation	.	Std Error Mean	0.03235728

Basic Statistical Measures

Location		Variability	
Mean	0.00000	Std Deviation	0.53070
Median	-0.12650	Variance	0.28164
Mode	-0.18650	Range	4.69806
		Interquartile Range	0.36900

NOTE: The mode displayed is the smallest of 4 modes with a count of 4.

Tests for Location: Mu0=0

Test	-Statistic-		-----p Value------	
Student's t	t	0	Pr > |t|	1.0000
Sign	M	-40.5	Pr >= |M|	<.0001
Signed Rank	S	-4884.5	Pr >= |S|	0.0001

Tests for Normality

Test	--Statistic---		-----p Value------	
Shapiro-Wilk	W	0.698635	Pr < W	<0.0001
Kolmogorov-Smirnov	D	0.177585	Pr > D	<0.0100
Cramer-von Mises	W-Sq	3.346828	Pr > W-Sq	<0.0050
Anderson-Darling	A-Sq	18.12	Pr > A-Sq	<0.0050

Quantiles (Definition 5)

Quantile	Estimate
100% Max	3.863500
99%	3.115441
95%	0.885441
90%	0.491884
75% Q3	0.112500
50% Median	-0.126500
25% Q1	-0.256500
10%	-0.384559
5%	-0.474559
1%	-0.784559
0% Min	-0.834559

Extreme Observations

------Lowest------		-----Highest-----	
Value	Obs	Value	Obs
-0.834559	39	1.34544	8
-0.784559	27	2.14544	62
-0.784559	24	3.11544	44

```
                 -0.774559        63         3.19544       11
                 -0.764559         5         3.86350      196


       PROC UNIVARIATE OUTPUT FOR RESIDUALS FROM GLM PROCEDURE USING FROG WEIGHT BY SEX      636

-------------------------------------------- Sex=M --------------------------------------------

                                    The UNIVARIATE Procedure
                                        Variable:  Resid

                        Histogram                             #           Boxplot
            3.9+*                                             1              *
               .
               .
               .*                                             2              *
               .
               .
               .*                                             1              *
               .
            1.5+
               .*                                             2              *
               .***                                           5              0
               .**                                            4              0
               .****                                          8              0
               .****                                          8              |
               .***********                                  21              |
               .*********************                        42           +--+--+
               .*****************************************    82           *-----*
               .*************************************        73           +-----+
               .******                                       12              |
               .****                                          7              |
           -0.9+*                                             1              0
                ----+----+----+----+----+----+----+----+-
                * may represent up to 2 counts


                                    The UNIVARIATE Procedure
                                        Variable:  Resid

                                     Normal Probability Plot
                    3.9+                                                     *
                       |
                       |
                       |                                                   * *
                       |
                       |
                       |
                       |                                                  *
                       |
                       |
                    1.5+
                       |                                                ** +++
                       |                                              ***++
                       |                                            **+
                       |                                        ++***
                       |                                    ++++***
                       |                                ++++ ****
                       |                            ++++******
                       |                        *********
                       |              ***********
                       |         *****    ++++
                       |* *****      ++++
                   -0.9+*       +++
```

```
+----+----+----+----+----+----+----+----+----+----+
    -2        -1         0        +1        +2
```

PROC UNIVARIATE OUTPUT FOR RESIDUALS FROM GLM PROCEDURE USING FROG WEIGHT BY SEX 638

```
                         The UNIVARIATE Procedure
                             Variable:  Resid

                             Schematic Plots

        |
      4 +
        |                       *
        |
        |           *
    3.5 +
        |           *
        |                       *
        |                       *
      3 +
        |           *
        |
        |
    2.5 +           *
        |
        |           *
        |                       *
      2 +
        |
        |
        |           *
    1.5 +           *
        |           *           *
        |           *           *
        |           *           0
      1 +           0           0
        |           0           0
        |           0           0
        |           0           0
    0.5 +           |           |
        |           |           |
        |           |           |
        |           |        +-----+
      0 +        +--+--+     |  +  |
        |        *-----*     *-----*
        |        +-----+     +-----+
        |           |           |
   -0.5 +           |           |
        |           |           |
        |           0           |
        |                       0
     -1 +
         ------------+-----------+-----------
    Sex              F           M
```

REGRESSION ANALYSIS OF WEIGHT OVER ATRAZINE CONCENTRATION 639

-- Sex=F --

The REG Procedure
Model: MODEL1
Dependent Variable: WEIGHT

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	1	0.06563	0.06563	1.96	0.2969
Error	2	0.06711	0.03356		
Corrected Total	3	0.13274			

Root MSE	0.18318	R-Square	0.4944
Dependent Mean	0.77747	Adj R-Sq	0.2416
Coeff Var	23.56145		

Parameter Estimates

Variable	DF	Parameter Estimate	Standard Error	t Value	Pr > |t|
Intercept	1	0.89246	0.12309	7.25	0.0185
ATRAZINE	1	-0.01278	0.00914	-1.40	0.2969

REGRESSION ANALYSIS OF WEIGHT OVER ATRAZINE CONCENTRATION 640

-- Sex=M --

The REG Procedure
Model: MODEL1
Dependent Variable: WEIGHT

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	1	0.07648	0.07648	1.25	0.3800
Error	2	0.12248	0.06124		
Corrected Total	3	0.19896			

Root MSE	0.24747	R-Square	0.3844
Dependent Mean	0.82417	Adj R-Sq	0.0766
Coeff Var	30.02629		

Parameter Estimates

Variable	DF	Parameter Estimate	Standard Error	t Value	Pr > |t|
Intercept	1	0.94830	0.16628	5.70	0.0294
ATRAZINE	1	-0.01379	0.01234	-1.12	0.3800

```
                  PLOT OF FROG LENGTH OVER ATRAZINE CONCENTRATION FOR MALE AND FEMALE FROGS          641

-------------------------------------------- Sex=F --------------------------------------------

                            Plot of LENGTH*ATRAZINE.  Symbol used is '*'.

LENGTH é
  21.5 ê
       é
       é
       é  *
       é
  21.0 ê
       é
       é
       é
       é
  20.5 ê
       é
       é
       é
       é
  20.0 ê
       é
       é
       é
       é
  19.5 ê
       é
       é
       é
       é
  19.0 ê
       é
       é                                *
       é
       é     *
  18.5 ê
       é
       é
       é
       é
  18.0 ê
       é
       é
       é
       é
  17.5 ê                                                                             *
       é
       èââêââêââêââêââêââêââêââêââêââêââêââêââêââêââêââêââêââêââêââêââêââêââêââêââêââêââ
          0  1  2  3  4  5  6  7  8  9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25

                                             ATRAZINE
```

```
           PLOT OF FROG LENGTH OVER ATRAZINE CONCENTRATION FOR MALE AND FEMALE FROGS             642

-------------------------------------------- Sex=M -------------------------------------------

                         Plot of LENGTH*ATRAZINE.  Symbol used is '*'.

    LENGTH é
           é
      22.5 ê
           é
           é
           é  *
      22.0 ê
           é
           é
           é
      21.5 ê
           é
           é
           é
      21.0 ê
           é
           é
           é
      20.5 ê
           é
           é
           é
      20.0 ê
           é
           é
           é
      19.5 ê
           é
           é
           é
      19.0 ê
           é                                *
           é     *
           é
      18.5 ê
           é
           é
           é                                                                             *
      18.0 ê
           é
           èâââêâââêâââêâââêâââêâââêâââêâââêâââêâââêâââêâââêâââêâââêâââêâââêâââêâââêâââêâââêâââêâââêâââêâââêâââêââ
              0   1   2   3   4   5   6   7   8   9  10  11  12  13  14  15  16  17  18  19  20  21  22  23  24  25

                                                    ATRAZINE
```

ANALYSIS OF VARIANCE FOR FROG LENGTH BETWEEN TREATMENT GROUPS BY SEX 643

-- Sex=F --

The GLM Procedure

Class Level Information

Class	Levels	Values
ATRAZINE	4	0 1 10 25

Number of observations 331

Dependent Variable: SVL

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	3	585.622241	195.207414	18.21	<.0001
Error	327	3505.727849	10.720880		
Corrected Total	330	4091.350091			

R-Square	Coeff Var	Root MSE	SVL Mean
0.143137	17.21578	3.274276	19.01903

Source	DF	Type I SS	Mean Square	F Value	Pr > F
ATRAZINE	3	585.6222415	195.2074138	18.21	<.0001

Source	DF	Type III SS	Mean Square	F Value	Pr > F
ATRAZINE	3	585.6222415	195.2074138	18.21	<.0001

ANALYSIS OF VARIANCE FOR FROG LENGTH BETWEEN TREATMENT GROUPS BY SEX 645

-- Sex=M --

The GLM Procedure

Class Level Information

Class	Levels	Values
ATRAZINE	4	0 1 10 25

Number of observations 269

Dependent Variable: SVL

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	3	640.258332	213.419444	21.98	<.0001
Error	265	2572.541817	9.707705		
Corrected Total	268	3212.800149			

R-Square	Coeff Var	Root MSE	SVL Mean
0.199284	16.01009	3.115719	19.46097

Source	DF	Type I SS	Mean Square	F Value	Pr > F
ATRAZINE	3	640.2583321	213.4194440	21.98	<.0001

Source	DF	Type III SS	Mean Square	F Value	Pr > F

ATRAZINE	3	640.2583321	213.4194440	21.98	<.0001

PROC UNIVARIATE OUTPUT FOR RESIDUALS FROM GLM PROCEDURE USING FROG LENGTH BY SEX 647

--- Sex=F ---

The UNIVARIATE Procedure
Variable: Resid

Moments

N	331	Sum Weights	331
Mean	0	Sum Observations	0
Std Deviation	3.25935848	Variance	10.6234177
Skewness	1.6487435	Kurtosis	4.17321286
Uncorrected SS	3505.72785	Corrected SS	3505.72785
Coeff Variation	.	Std Error Mean	0.17915054

Basic Statistical Measures

Location		Variability	
Mean	0.00000	Std Deviation	3.25936
Median	-0.62444	Variance	10.62342
Mode	-1.42444	Range	22.72940
		Interquartile Range	2.77060

NOTE: The mode displayed is the smallest of 2 modes with a count of 6.

Tests for Location: Mu0=0

Test	-Statistic-		-----p Value------	
Student's t	t	0	Pr > |t|	1.0000
Sign	M	-36.5	Pr >= |M|	<.0001
Signed Rank	S	-5490	Pr >= |S|	0.0015

Tests for Normality

Test	--Statistic---		-----p Value------	
Shapiro-Wilk	W	0.871872	Pr < W	<0.0001
Kolmogorov-Smirnov	D	0.1515	Pr > D	<0.0100
Cramer-von Mises	W-Sq	2.170587	Pr > W-Sq	<0.0050
Anderson-Darling	A-Sq	12.12968	Pr > A-Sq	<0.0050

Quantiles (Definition 5)

Quantile	Estimate
100% Max	15.775556
99%	11.395062
95%	6.729268
90%	3.646154
75% Q3	0.946154
50% Median	-0.624444
25% Q1	-1.824444
10%	-3.004938
5%	-4.104938
1%	-5.670732
0% Min	-6.953846

Extreme Observations

------Lowest-----		-----Highest-----	
Value	Obs	Value	Obs
-6.95385	83	10.4756	248
-6.17073	1	11.3951	279

-5.87073	6	13.5293	71
-5.67073	38	13.9293	13
-5.57073	5	15.7756	250

```
      PROC UNIVARIATE OUTPUT FOR RESIDUALS FROM GLM PROCEDURE USING FROG LENGTH BY SEX       649

------------------------------------------- Sex=F ------------------------------------------

                                   The UNIVARIATE Procedure
                                       Variable:  Resid

                          Histogram                         #             Boxplot
            15.5+*                                          1                *
                .
                .*                                          2                *
                .
                .*                                          1                *
                .*                                          2                *
                .**                                         3                *
                .*                                          2                0
                .***                                        5                0
                .***                                        6                0
                .***                                        6                0
             4.5+**                                         3                |
                .*****                                     10                |
                .********                                  15                |
                .************                              23                |
                .*************************                 50             +--+--+
                .******************************            59             *-----*
                .************************************      71             +-----+
                .*******************                       38                |
                .********                                  16                |
                .******                                    11                |
                .***                                        5                |
            -6.5+*                                          2                0
                 ----+----+----+----+----+----+----+-
                 * may represent up to 2 counts


                                   The UNIVARIATE Procedure
                                       Variable:  Resid

                                     Normal Probability Plot
                15.5+                                                          *
                    |
                    |                                                         **
                    |
                    |                                                        *
                    |                                                      **
                    |                                                     ***
                    |                                                     *    +
                    |                                                   **  +++
                    |                                                 **  +++
                    |                                                *****+
                 4.5+                                                **+
                    |                                             ++**
                    |                                          +++**
                    |                                       +++****
                    |                                   +++*****
                    |                                +******
                    |                          *******
                    |                     ******+
                    |                  ****+++
                    |              *****+++
                    | ****   +++
                -6.5+*    +++
                     +----+----+----+----+----+----+----+----+----+----+
                         -2        -1         0        +1        +2
```

PROC UNIVARIATE OUTPUT FOR RESIDUALS FROM GLM PROCEDURE USING FROG LENGTH BY SEX 651

--- Sex=M ---

The UNIVARIATE Procedure
Variable: Resid

Moments

N	269	Sum Weights	269
Mean	0	Sum Observations	0
Std Deviation	3.09823121	Variance	9.59903663
Skewness	1.47756279	Kurtosis	4.18236889
Uncorrected SS	2572.54182	Corrected SS	2572.54182
Coeff Variation	.	Std Error Mean	0.18890249

Basic Statistical Measures

Location		Variability	
Mean	0.00000	Std Deviation	3.09823
Median	-0.57059	Variance	9.59904
Mode	-1.68056	Range	22.72892
		Interquartile Range	2.85713

Tests for Location: Mu0=0

Test	-Statistic-		-----p Value------	
Student's t	t	0	Pr > |t|	1.0000
Sign	M	-29.5	Pr >= |M|	0.0004
Signed Rank	S	-2839.5	Pr >= |S|	0.0259

Tests for Normality

Test	--Statistic---		-----p Value------	
Shapiro-Wilk	W	0.903548	Pr < W	<0.0001
Kolmogorov-Smirnov	D	0.137053	Pr > D	<0.0100
Cramer-von Mises	W-Sq	1.231739	Pr > W-Sq	<0.0050
Anderson-Darling	A-Sq	6.644115	Pr > A-Sq	<0.0050

Quantiles (Definition 5)

Quantile	Estimate
100% Max	15.858333
99%	12.229412
95%	6.029412
90%	4.162319
75% Q3	1.119444
50% Median	-0.570588
25% Q1	-1.737681
10%	-3.080556
5%	-3.880556
1%	-5.870588
0% Min	-6.870588

Extreme Observations

------Lowest-----		------Highest-----	
Value	Obs	Value	Obs
-6.87059	27	8.45833	183
-6.27059	63	8.52941	62

-5.87059	24	12.22941	44
-5.77059	39	13.32941	11
-5.37059	5	15.85833	196

```
         PROC UNIVARIATE OUTPUT FOR RESIDUALS FROM GLM PROCEDURE USING FROG LENGTH BY SEX      653

--------------------------------------------- Sex=M ---------------------------------------------

                                  The UNIVARIATE Procedure
                                      Variable:  Resid

                       Histogram                        #             Boxplot
            15.5+*                                       1                *
                .
                .*                                       1                *
                .*                                       1                *
                .
                .
                .
                .**                                      4                0
                .*                                       2                0
                .***                                     5                0
                .**                                      4                |
             4.5+*****                                  10                |
                .******                                 11                |
                .******                                 12                |
                .***********                            21             +-----+
                .*****************                      33             |  +  |
                .**************************             51             *-----*
                .****************************           56             +-----+
                .***************                        30                |
                .********                               16                |
                .***                                     6                |
                .**                                      3                |
            -6.5+*                                       2                0
                 ----+----+----+----+----+---
                 * may represent up to 2 counts


                                  The UNIVARIATE Procedure
                                      Variable:  Resid

                                     Normal Probability Plot
              15.5+                                                        *
                  |
                  |                                                        *
                  |                                                      *
                  |
                  |
                  |
                  |                                                   ***
                  |                                                  *   +++
                  |                                               *** +++
                  |                                              **+++
               4.5+                                            ***+
                  |                                         +**
                  |                                      ++**
                  |                                   ++****
                  |                               +++****
                  |                            +******
                  |                       *******
                  |                  ******+
                  |              *****+++
                  |           *** ++++
                  |        *** +++
              -6.5+*    +++
                   +----+----+----+----+----+----+----+----+----+----+
                        -2        -1         0        +1        +2
```

PROC UNIVARIATE OUTPUT FOR RESIDUALS FROM GLM PROCEDURE USING FROG LENGTH BY SEX 655

The UNIVARIATE Procedure
Variable: Resid

Schematic Plots

```
       |
  17.5 +
       |
       |
       |            *           *
    15 +
       |
       |            *
       |                        *
  12.5 +                        *
       |
       |            *
       |            *
    10 +            *
       |            *
       |            0           0
       |            0           0
   7.5 +            0           0
       |            0
       |            0           0
       |            0           |
     5 +            0           |
       |            |           |
       |            |           |
       |            |           |
   2.5 +            |           |
       |            |           |
       |         +-----+     +-----+
       |         |     |     |     |
     0 +         |  +  |     |  +  |
       |         *-----*     *-----*
       |         |     |     |     |
       |         +-----+     +-----+
  -2.5 +            |           |
       |            |           |
       |            |           |
       |            |           |
    -5 +            |           |
       |            |           |
       |            0           0
       |            0           0
  -7.5 +
        ------------+-----------+----------
   Sex                 F           M
```

REGRESSION ANALYSIS OF LENGTH OVER ATRAZINE CONCENTRATION 656

--- Sex=F ---

The REG Procedure
Model: MODEL1
Dependent Variable: LENGTH

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	1	3.99283	3.99283	2.51	0.2541
Error	2	3.18361	1.59180		
Corrected Total	3	7.17643			

Root MSE	1.26167	R-Square	0.5564
Dependent Mean	19.01349	Adj R-Sq	0.3346
Coeff Var	6.63564		

Parameter Estimates

Variable	DF	Parameter Estimate	Standard Error	t Value	Pr > |t|
Intercept	1	19.91044	0.84775	23.49	0.0018
ATRAZINE	1	-0.09966	0.06293	-1.58	0.2541

REGRESSION ANALYSIS OF LENGTH OVER ATRAZINE CONCENTRATION 657

--- Sex=M ---

The REG Procedure
Model: MODEL1
Dependent Variable: LENGTH

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	1	3.94737	3.94737	1.45	0.3522
Error	2	5.46029	2.73015		
Corrected Total	3	9.40766			

Root MSE	1.65232	R-Square	0.4196
Dependent Mean	19.45762	Adj R-Sq	0.1294
Coeff Var	8.49187		

Parameter Estimates

Variable	DF	Parameter Estimate	Standard Error	t Value	Pr > |t|
Intercept	1	20.34946	1.11024	18.33	0.0030
ATRAZINE	1	-0.09909	0.08241	-1.20	0.3522

ANOVA FOR FROG LENGTH BETWEEN TREATMENTS 658

--- Sex=F ---

The ANOVA Procedure

Class Level Information

Class	Levels	Values
ATRAZINE	4	0 1 10 25

Number of observations 10

Dependent Variable: LENGTH

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	3	24.31994787	8.10664929	6.17	0.0290
Error	6	7.88332214	1.31388702		
Corrected Total	9	32.20327001			

R-Square	Coeff Var	Root MSE	LENGTH Mean
0.755201	6.043316	1.146249	18.96722

Source	DF	Anova SS	Mean Square	F Value	Pr > F
ATRAZINE	3	24.31994787	8.10664929	6.17	0.0290

Levene's Test for Homogeneity of LENGTH Variance
ANOVA of Squared Deviations from Group Means

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
ATRAZINE	1	1.4602	1.4602	2.90	0.1640
Error	4	2.0163	0.5041		

Bartlett's Test for Homogeneity of LENGTH Variance

Source	DF	Chi-Square	Pr > ChiSq
ATRAZINE	3	1.6226	0.6543

Dunnett's t Tests for LENGTH

NOTE: This test controls the Type I experimentwise error for comparisons of all treatments against a control.

Alpha	0.05
Error Degrees of Freedom	6
Error Mean Square	1.313887
Critical Value of Dunnett's t	3.12383

Comparisons significant at the 0.05 level are indicated by ***.

ATRAZINE Comparison	Difference Between Means	Simultaneous 95% Confidence Limits		
10 - 0	-2.2703	-5.5390	0.9984	
1 - 0	-2.5773	-5.8460	0.6915	
25 - 0	-3.9812	-6.9049	-1.0576	***

ANOVA FOR FROG LENGTH BETWEEN TREATMENTS 662

-- Sex=M --

The ANOVA Procedure

Class Level Information

Class	Levels	Values
ATRAZINE	4	0 1 10 25

Number of observations 10

Dependent Variable: LENGTH

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	3	32.02748490	10.67582830	14.25	0.0039
Error	6	4.49480602	0.74913434		
Corrected Total	9	36.52229092			

R-Square	Coeff Var	Root MSE	LENGTH Mean
0.876930	4.402765	0.865525	19.65868

Source	DF	Anova SS	Mean Square	F Value	Pr > F
ATRAZINE	3	32.02748490	10.67582830	14.25	0.0039

Levene's Test for Homogeneity of LENGTH Variance
ANOVA of Squared Deviations from Group Means

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
ATRAZINE	1	0.4820	0.4820	3.52	0.1337
Error	4	0.5470	0.1368		

Bartlett's Test for Homogeneity of LENGTH Variance

Source	DF	Chi-Square	Pr > ChiSq
ATRAZINE	3	3.1456	0.3697

Dunnett's t Tests for LENGTH

NOTE: This test controls the Type I experimentwise error for comparisons of all treatments against a control.

Alpha	0.05
Error Degrees of Freedom	6
Error Mean Square	0.749134
Critical Value of Dunnett's t	3.12383

Comparisons significant at the 0.05 level are indicated by ***.

ATRAZINE Comparison	Difference Between Means	Simultaneous 95% Confidence Limits		
10 - 0	-3.5532	-6.0214	-1.0851	***
1 - 0	-3.5778	-6.0460	-1.1096	***

```
25   - 0          -4.2449      -6.4525  -2.0373  ***
```

NONPARAMETRIC COMPARISON OF FROG LENGTH ACROSS TREATMENTS 666

--- Sex=F ---

The NPAR1WAY Procedure

Wilcoxon Scores (Rank Sums) for Variable LENGTH
Classified by Variable ATRAZINE

ATRAZINE	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
0	3	27.0	16.50	4.387482	9.000000
1	2	10.0	11.00	3.829708	5.000000
10	2	10.0	11.00	3.829708	5.000000
25	3	8.0	16.50	4.387482	2.666667

Kruskal-Wallis Test

Chi-Square	6.7455
DF	3
Pr > Chi-Square	0.0805

Median Scores (Number of Points Above Median) for Variable LENGTH
Classified by Variable ATRAZINE

ATRAZINE	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
0	3	3.0	1.50	0.763763	1.00
1	2	1.0	1.00	0.666667	0.50
10	2	1.0	1.00	0.666667	0.50
25	3	0.0	1.50	0.763763	0.00

Median One-Way Analysis

Chi-Square	5.4000
DF	3
Pr > Chi-Square	0.1447

--- Sex=M ---

The NPAR1WAY Procedure

Wilcoxon Scores (Rank Sums) for Variable LENGTH
Classified by Variable ATRAZINE

ATRAZINE	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
0	3	27.0	16.50	4.387482	9.000000
1	2	8.0	11.00	3.829708	4.000000
10	2	10.0	11.00	3.829708	5.000000
25	3	10.0	16.50	4.387482	3.333333

Kruskal-Wallis Test

Chi-Square	6.0909
DF	3
Pr > Chi-Square	0.1073

Median Scores (Number of Points Above Median) for Variable LENGTH
Classified by Variable ATRAZINE

ATRAZINE	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
0	3	3.0	1.50	0.763763	1.00
1	2	1.0	1.00	0.666667	0.50
10	2	1.0	1.00	0.666667	0.50
25	3	0.0	1.50	0.763763	0.00

Median One-Way Analysis

Chi-Square	5.4000
DF	3
Pr > Chi-Square	0.1447

ANOVA FOR FROG WEIGHT BETWEEN TREATMENTS

--- Sex=F ---

The ANOVA Procedure

Class Level Information

Class	Levels	Values
ATRAZINE	4	0 1 10 25

Number of observations 10

Dependent Variable: WEIGHT

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	3	0.47376094	0.15792031	7.93	0.0165
Error	6	0.11947701	0.01991283		
Corrected Total	9	0.59323795			

R-Square	Coeff Var	Root MSE	WEIGHT Mean
0.798602	17.94657	0.141113	0.786294

Source	DF	Anova SS	Mean Square	F Value	Pr > F
ATRAZINE	3	0.47376094	0.15792031	7.93	0.0165

Levene's Test for Homogeneity of WEIGHT Variance
ANOVA of Squared Deviations from Group Means

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
ATRAZINE	1	0.000095	0.000095	1.45	0.2953
Error	4	0.000264	0.000066		

Bartlett's Test for Homogeneity of WEIGHT Variance

Source	DF	Chi-Square	Pr > ChiSq
ATRAZINE	3	0.9200	0.8206

Dunnett's t Tests for WEIGHT

NOTE: This test controls the Type I experimentwise error for comparisons of all treatments against a control.

Alpha	0.05
Error Degrees of Freedom	6
Error Mean Square	0.019913
Critical Value of Dunnett's t	3.12383

Comparisons significant at the 0.05 level are indicated by ***.

ATRAZINE Comparison	Difference Between Means	Simultaneous 95% Confidence Limits		
10 - 0	-0.3623	-0.7647	0.0401	
1 - 0	-0.3974	-0.7998	0.0050	
25 - 0	-0.5445	-0.9044	-0.1846	***

--- Sex=M ---

The ANOVA Procedure

Class Level Information

Class	Levels	Values
ATRAZINE	4	0 1 10 25

Number of observations 10

Dependent Variable: WEIGHT

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	3	0.73743515	0.24581172	9.66	0.0103
Error	6	0.15260050	0.02543342		
Corrected Total	9	0.89003565			

R-Square	Coeff Var	Root MSE	WEIGHT Mean
0.828546	18.48632	0.159479	0.862684

Source	DF	Anova SS	Mean Square	F Value	Pr > F
ATRAZINE	3	0.73743515	0.24581172	9.66	0.0103

Levene's Test for Homogeneity of WEIGHT Variance
ANOVA of Squared Deviations from Group Means

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
ATRAZINE	1	0.00150	0.00150	4.00	0.1161
Error	4	0.00150	0.000376		

Bartlett's Test for Homogeneity of WEIGHT Variance

Source	DF	Chi-Square	Pr > ChiSq
ATRAZINE	3	12.4660	0.0059

Dunnett's t Tests for WEIGHT

NOTE: This test controls the Type I experimentwise error for comparisons of all treatments against a control.

Alpha	0.05
Error Degrees of Freedom	6
Error Mean Square	0.025433
Critical Value of Dunnett's t	3.12383

Comparisons significant at the 0.05 level are indicated by ***.

ATRAZINE Comparison	Difference Between Means	Simultaneous 95% Confidence Limits

```
10    - 0        -0.5352    -0.9900 -0.0805  ***
1     - 0        -0.5662    -1.0210 -0.1114  ***
25    - 0        -0.6358    -1.0426 -0.2290  ***
```

-- Sex=F --

The NPAR1WAY Procedure

Wilcoxon Scores (Rank Sums) for Variable WEIGHT
Classified by Variable ATRAZINE

ATRAZINE	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
0	3	27.0	16.50	4.387482	9.00
1	2	8.0	11.00	3.829708	4.00
10	2	11.0	11.00	3.829708	5.50
25	3	9.0	16.50	4.387482	3.00

Kruskal-Wallis Test

Chi-Square	6.5455
DF	3
Pr > Chi-Square	0.0879

Median Scores (Number of Points Above Median) for Variable WEIGHT
Classified by Variable ATRAZINE

ATRAZINE	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
0	3	3.0	1.50	0.763763	1.00
1	2	1.0	1.00	0.666667	0.50
10	2	1.0	1.00	0.666667	0.50
25	3	0.0	1.50	0.763763	0.00

Median One-Way Analysis

Chi-Square	5.4000
DF	3
Pr > Chi-Square	0.1447

NONPARAMETRIC COMPARISON OF FROG WEIGHT ACROSS TREATMENTS BY SEX 680

--- Sex=M ---

The NPAR1WAY Procedure

Wilcoxon Scores (Rank Sums) for Variable WEIGHT
Classified by Variable ATRAZINE

ATRAZINE	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
0	3	27.0	16.50	4.387482	9.00
1	2	7.0	11.00	3.829708	3.50
10	2	9.0	11.00	3.829708	4.50
25	3	12.0	16.50	4.387482	4.00

Kruskal-Wallis Test

Chi-Square	5.8364
DF	3
Pr > Chi-Square	0.1198

Median Scores (Number of Points Above Median) for Variable WEIGHT
Classified by Variable ATRAZINE

ATRAZINE	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
0	3	3.0	1.50	0.763763	1.00
1	2	1.0	1.00	0.666667	0.50
10	2	1.0	1.00	0.666667	0.50
25	3	0.0	1.50	0.763763	0.00

Median One-Way Analysis

Chi-Square	5.4000
DF	3
Pr > Chi-Square	0.1447

--- ATRAZINE=0 ---

The ANOVA Procedure

Class Level Information

Class	Levels	Values
Sex	2	F M

Number of observations 6

Dependent Variable: WEIGHT

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	1	0.04444191	0.04444191	1.64	0.2697
Error	4	0.10848814	0.02712203		
Corrected Total	5	0.15293005			

R-Square	Coeff Var	Root MSE	WEIGHT Mean
0.290603	13.86664	0.164688	1.187654

Source	DF	Anova SS	Mean Square	F Value	Pr > F
Sex	1	0.04444191	0.04444191	1.64	0.2697

Levene's Test for Homogeneity of WEIGHT Variance
ANOVA of Squared Deviations from Group Means

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Sex	1	0.00111	0.00111	2.89	0.1646
Error	4	0.00153	0.000384		

Bartlett's Test for Homogeneity of WEIGHT Variance

Source	DF	Chi-Square	Pr > ChiSq
Sex	1	1.3299	0.2488

Dunnett's t Tests for WEIGHT

NOTE: This test controls the Type I experimentwise error for comparisons of all treatments against a control.

Alpha	0.05
Error Degrees of Freedom	4
Error Mean Square	0.027122
Critical Value of Dunnett's t	2.77630
Minimum Significant Difference	0.3733

Comparisons significant at the 0.05 level are indicated by ***.

Sex Comparison	Difference Between Means	Simultaneous 95% Confidence Limits

```
M    - F          0.1721    -0.2012  0.5454
```

ANOVA FOR WEIGHT BETWEEN SEXES

-- ATRAZINE=1 --

The ANOVA Procedure

Class Level Information

Class	Levels	Values
Sex	2	F M

Number of observations 4

Dependent Variable: WEIGHT

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	1	0.00001069	0.00001069	0.00	0.9872
Error	2	0.06567277	0.03283639		
Corrected Total	3	0.06568346			

R-Square	Coeff Var	Root MSE	WEIGHT Mean
0.000163	25.67177	0.181208	0.705865

Source	DF	Anova SS	Mean Square	F Value	Pr > F
Sex	1	0.00001069	0.00001069	0.00	0.9872

Levene's Test for Homogeneity of WEIGHT Variance
ANOVA of Squared Deviations from Group Means

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Sex	0	0	.	.	.
Error	0	0	.		

Bartlett's Test for Homogeneity of WEIGHT Variance

Source	DF	Chi-Square	Pr > ChiSq
Sex	1	0.0108	0.9171

Dunnett's t Tests for WEIGHT

NOTE: This test controls the Type I experimentwise error for comparisons of all treatments against a control.

Alpha	0.05
Error Degrees of Freedom	2
Error Mean Square	0.032836
Critical Value of Dunnett's t	4.30263
Minimum Significant Difference	0.7797

Comparisons significant at the 0.05 level are indicated by ***.

Sex	Difference Between	Simultaneous 95%

Comparison	Means	Confidence Limits	
M - F	0.003269	-0.776402	0.782940

-- ATRAZINE=10 --

The ANOVA Procedure

Class Level Information

Class	Levels	Values
Sex	2	F M

Number of observations 4

Dependent Variable: WEIGHT

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	1	0.00000059	0.00000059	0.00	0.9969
Error	2	0.06049619	0.03024810		
Corrected Total	3	0.06049678			

R-Square	Coeff Var	Root MSE	WEIGHT Mean
0.000010	23.53874	0.173920	0.738866

Source	DF	Anova SS	Mean Square	F Value	Pr > F
Sex	1	5.9017464E-7	5.9017464E-7	0.00	0.9969

Levene's Test for Homogeneity of WEIGHT Variance
ANOVA of Squared Deviations from Group Means

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Sex	0	0	.	.	.
Error	0	0	.		

Bartlett's Test for Homogeneity of WEIGHT Variance

Source	DF	Chi-Square	Pr > ChiSq
Sex	1	0.00127	0.9716

Dunnett's t Tests for WEIGHT

NOTE: This test controls the Type I experimentwise error for comparisons of all treatments against a control.

Alpha	0.05
Error Degrees of Freedom	2
Error Mean Square	0.030248
Critical Value of Dunnett's t	4.30263
Minimum Significant Difference	0.7483

Comparisons significant at the 0.05 level are indicated by ***.

Sex	Difference Between	Simultaneous 95%

Comparison	Means	Confidence Limits	
M - F	-0.0007682	-0.7490802	0.7475438

ANOVA FOR WEIGHT BETWEEN SEXES 694

-- ATRAZINE=25 --

The ANOVA Procedure

Class Level Information

Class	Levels	Values
Sex	2	F M

Number of observations 6

Dependent Variable: WEIGHT

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	1	0.00980227	0.00980227	1.05	0.3639
Error	4	0.03742040	0.00935510		
Corrected Total	5	0.04722267			

R-Square	Coeff Var	Root MSE	WEIGHT Mean
0.207575	16.18803	0.096722	0.597489

Source	DF	Anova SS	Mean Square	F Value	Pr > F
Sex	1	0.00980227	0.00980227	1.05	0.3639

Levene's Test for Homogeneity of WEIGHT Variance
ANOVA of Squared Deviations from Group Means

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Sex	1	0.000233	0.000233	4.00	0.1162
Error	4	0.000233	0.000058		

Bartlett's Test for Homogeneity of WEIGHT Variance

Source	DF	Chi-Square	Pr > ChiSq
Sex	1	11.6016	0.0007

Dunnett's t Tests for WEIGHT

NOTE: This test controls the Type I experimentwise error for comparisons of all treatments against a control.

Alpha	0.05
Error Degrees of Freedom	4
Error Mean Square	0.009355
Critical Value of Dunnett's t	2.77630
Minimum Significant Difference	0.2193

Comparisons significant at the 0.05 level are indicated by ***.

Sex	Difference Between	Simultaneous 95%

Comparison	Means	Confidence Limits	
M - F	0.08084	-0.13841	0.30009

NONPARAMETRIC COMPARISON OF FROG WEIGHT BETWEEN SEXES FOR EACH EXPOSURE GROUP 698

-- ATRAZINE=0 --

The NPAR1WAY Procedure

Wilcoxon Scores (Rank Sums) for Variable WEIGHT
Classified by Variable Sex

Sex	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
F	3	8.0	10.50	2.291288	2.666667
M	3	13.0	10.50	2.291288	4.333333

Wilcoxon Two-Sample Test

Statistic	8.0000
Normal Approximation	
Z	-0.8729
One-Sided Pr < Z	0.1914
Two-Sided Pr > |Z|	0.3827
t Approximation	
One-Sided Pr < Z	0.2113
Two-Sided Pr > |Z|	0.4227

Z includes a continuity correction of 0.5.

Kruskal-Wallis Test

Chi-Square	1.1905
DF	1
Pr > Chi-Square	0.2752

Median Scores (Number of Points Above Median) for Variable WEIGHT
Classified by Variable Sex

Sex	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
F	3	1.0	1.50	0.670820	0.333333
M	3	2.0	1.50	0.670820	0.666667

Median Two-Sample Test

Statistic	1.0000
Z	-0.7454
One-Sided Pr < Z	0.2280
Two-Sided Pr > |Z|	0.4561

Median One-Way Analysis

Chi-Square	0.5556
DF	1
Pr > Chi-Square	0.4561

--- ATRAZINE=1 ---

The NPAR1WAY Procedure

Wilcoxon Scores (Rank Sums) for Variable WEIGHT
Classified by Variable Sex

Sex	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
F	2	5.0	5.0	1.290994	2.50
M	2	5.0	5.0	1.290994	2.50

Wilcoxon Two-Sample Test

Statistic	5.0000
Normal Approximation	
Z	0.0000
One-Sided Pr < Z	0.5000
Two-Sided Pr > |Z|	1.0000
t Approximation	
One-Sided Pr < Z	0.5000
Two-Sided Pr > |Z|	1.0000

Z includes a continuity correction of 0.5.

Kruskal-Wallis Test

Chi-Square	0.0000
DF	1
Pr > Chi-Square	1.0000

Median Scores (Number of Points Above Median) for Variable WEIGHT
Classified by Variable Sex

Sex	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
F	2	1.0	1.0	0.577350	0.50
M	2	1.0	1.0	0.577350	0.50

Median Two-Sample Test

Statistic	1.0000
Z	0.0000
One-Sided Pr < Z	0.5000
Two-Sided Pr > |Z|	1.0000

Median One-Way Analysis

Chi-Square	0.0000
DF	1
Pr > Chi-Square	1.0000

NONPARAMETRIC COMPARISON OF FROG WEIGHT BETWEEN SEXES FOR EACH EXPOSURE GROUP 702

-- ATRAZINE=10 --

The NPAR1WAY Procedure

Wilcoxon Scores (Rank Sums) for Variable WEIGHT
Classified by Variable Sex

Sex	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
F	2	5.0	5.0	1.290994	2.50
M	2	5.0	5.0	1.290994	2.50

Wilcoxon Two-Sample Test

Statistic	5.0000
Normal Approximation	
Z	0.0000
One-Sided Pr < Z	0.5000
Two-Sided Pr > |Z|	1.0000
t Approximation	
One-Sided Pr < Z	0.5000
Two-Sided Pr > |Z|	1.0000

Z includes a continuity correction of 0.5.

Kruskal-Wallis Test

Chi-Square	0.0000
DF	1
Pr > Chi-Square	1.0000

The NPAR1WAY Procedure

Median Scores (Number of Points Above Median) for Variable WEIGHT
Classified by Variable Sex

Sex	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
F	2	1.0	1.0	0.577350	0.50
M	2	1.0	1.0	0.577350	0.50

Median Two-Sample Test

Statistic	1.0000
Z	0.0000
One-Sided Pr < Z	0.5000
Two-Sided Pr > |Z|	1.0000

Median One-Way Analysis

Chi-Square	0.0000
DF	1
Pr > Chi-Square	1.0000

-- ATRAZINE=25 --

The NPAR1WAY Procedure

Wilcoxon Scores (Rank Sums) for Variable WEIGHT
Classified by Variable Sex

Sex	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
F	3	9.0	10.50	2.291288	3.0
M	3	12.0	10.50	2.291288	4.0

Wilcoxon Two-Sample Test

Statistic	9.0000
Normal Approximation	
Z	-0.4364
One-Sided Pr < Z	0.3313
Two-Sided Pr > |Z|	0.6625
t Approximation	
One-Sided Pr < Z	0.3404
Two-Sided Pr > |Z|	0.6807

Z includes a continuity correction of 0.5.

Kruskal-Wallis Test

Chi-Square	0.4286
DF	1
Pr > Chi-Square	0.5127

The NPAR1WAY Procedure

Median Scores (Number of Points Above Median) for Variable WEIGHT
Classified by Variable Sex

Sex	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
F	3	1.0	1.50	0.670820	0.333333
M	3	2.0	1.50	0.670820	0.666667

Median Two-Sample Test

Statistic	1.0000
Z	-0.7454
One-Sided Pr < Z	0.2280
Two-Sided Pr > |Z|	0.4561

Median One-Way Analysis

Chi-Square	0.5556
DF	1
Pr > Chi-Square	0.4561

ANOVA FOR LENGTH BETWEEN SEXES

-- ATRAZINE=0 --

The ANOVA Procedure

Class Level Information

Class	Levels	Values
Sex	2	F M

Number of observations 6

Dependent Variable: WEIGHT

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	1	0.04444191	0.04444191	1.64	0.2697
Error	4	0.10848814	0.02712203		
Corrected Total	5	0.15293005			

R-Square	Coeff Var	Root MSE	WEIGHT Mean
0.290603	13.86664	0.164688	1.187654

Source	DF	Anova SS	Mean Square	F Value	Pr > F
Sex	1	0.04444191	0.04444191	1.64	0.2697

Dunnett's t Tests for WEIGHT

NOTE: This test controls the Type I experimentwise error for comparisons of all treatments against a control.

Alpha	0.05
Error Degrees of Freedom	4
Error Mean Square	0.027122
Critical Value of Dunnett's t	2.77630
Minimum Significant Difference	0.3733

Comparisons significant at the 0.05 level are indicated by ***.

Sex Comparison	Difference Between Means	Simultaneous 95% Confidence Limits	
M - F	0.1721	-0.2012	0.5454

ANOVA FOR LENGTH BETWEEN SEXES 709

-- ATRAZINE=1 --

The ANOVA Procedure

Class Level Information

Class	Levels	Values
Sex	2	F M

Number of observations 4

Dependent Variable: WEIGHT

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	1	0.00001069	0.00001069	0.00	0.9872
Error	2	0.06567277	0.03283639		
Corrected Total	3	0.06568346			

R-Square	Coeff Var	Root MSE	WEIGHT Mean
0.000163	25.67177	0.181208	0.705865

Source	DF	Anova SS	Mean Square	F Value	Pr > F
Sex	1	0.00001069	0.00001069	0.00	0.9872

Dunnett's t Tests for WEIGHT

NOTE: This test controls the Type I experimentwise error for comparisons of all treatments against a control.

Alpha	0.05
Error Degrees of Freedom	2
Error Mean Square	0.032836
Critical Value of Dunnett's t	4.30263
Minimum Significant Difference	0.7797

Comparisons significant at the 0.05 level are indicated by ***.

Sex Comparison	Difference Between Means	Simultaneous 95% Confidence Limits	
M - F	0.003269	-0.776402	0.782940

ANOVA FOR LENGTH BETWEEN SEXES 712

-- ATRAZINE=10 --

The ANOVA Procedure

Class Level Information

Class	Levels	Values
Sex	2	F M

Number of observations 4

Dependent Variable: WEIGHT

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	1	0.00000059	0.00000059	0.00	0.9969
Error	2	0.06049619	0.03024810		
Corrected Total	3	0.06049678			

R-Square	Coeff Var	Root MSE	WEIGHT Mean
0.000010	23.53874	0.173920	0.738866

Source	DF	Anova SS	Mean Square	F Value	Pr > F
Sex	1	5.9017464E-7	5.9017464E-7	0.00	0.9969

Dunnett's t Tests for WEIGHT

NOTE: This test controls the Type I experimentwise error for comparisons of all treatments against a control.

Alpha	0.05
Error Degrees of Freedom	2
Error Mean Square	0.030248
Critical Value of Dunnett's t	4.30263
Minimum Significant Difference	0.7483

Comparisons significant at the 0.05 level are indicated by ***.

Sex Comparison	Difference Between Means	Simultaneous 95% Confidence Limits	
M - F	-0.0007682	-0.7490802	0.7475438

ANOVA FOR LENGTH BETWEEN SEXES

-- ATRAZINE=25 --

The ANOVA Procedure

Class Level Information

Class	Levels	Values
Sex	2	F M

Number of observations 6

Dependent Variable: WEIGHT

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	1	0.00980227	0.00980227	1.05	0.3639
Error	4	0.03742040	0.00935510		
Corrected Total	5	0.04722267			

R-Square	Coeff Var	Root MSE	WEIGHT Mean
0.207575	16.18803	0.096722	0.597489

Source	DF	Anova SS	Mean Square	F Value	Pr > F
Sex	1	0.00980227	0.00980227	1.05	0.3639

Dunnett's t Tests for WEIGHT

NOTE: This test controls the Type I experimentwise error for comparisons of all treatments against a control.

Alpha	0.05
Error Degrees of Freedom	4
Error Mean Square	0.009355
Critical Value of Dunnett's t	2.77630
Minimum Significant Difference	0.2193

Comparisons significant at the 0.05 level are indicated by ***.

Sex Comparison	Difference Between Means	Simultaneous 95% Confidence Limits	
M - F	0.08084	-0.13841	0.30009

NONPARAMETRIC COMPARISON OF FROG LENGTH BETWEEN SEXES FOR EACH EXPOSURE GROUP 718

--- ATRAZINE=0 ---

The NPAR1WAY Procedure

Wilcoxon Scores (Rank Sums) for Variable WEIGHT
Classified by Variable Sex

Sex	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
F	3	8.0	10.50	2.291288	2.666667
M	3	13.0	10.50	2.291288	4.333333

Wilcoxon Two-Sample Test

Statistic	8.0000
Normal Approximation	
Z	-0.8729
One-Sided Pr < Z	0.1914
Two-Sided Pr > |Z|	0.3827
t Approximation	
One-Sided Pr < Z	0.2113
Two-Sided Pr > |Z|	0.4227

Z includes a continuity correction of 0.5.

Kruskal-Wallis Test

Chi-Square	1.1905
DF	1
Pr > Chi-Square	0.2752

Median Scores (Number of Points Above Median) for Variable WEIGHT
Classified by Variable Sex

Sex	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
F	3	1.0	1.50	0.670820	0.333333
M	3	2.0	1.50	0.670820	0.666667

Median Two-Sample Test

Statistic	1.0000
Z	-0.7454
One-Sided Pr < Z	0.2280
Two-Sided Pr > |Z|	0.4561

Median One-Way Analysis

Chi-Square	0.5556
DF	1
Pr > Chi-Square	0.4561

-- ATRAZINE=1 --

The NPAR1WAY Procedure

Wilcoxon Scores (Rank Sums) for Variable WEIGHT
Classified by Variable Sex

Sex	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
F	2	5.0	5.0	1.290994	2.50
M	2	5.0	5.0	1.290994	2.50

Wilcoxon Two-Sample Test

Statistic	5.0000
Normal Approximation	
Z	0.0000
One-Sided Pr < Z	0.5000
Two-Sided Pr > |Z|	1.0000
t Approximation	
One-Sided Pr < Z	0.5000
Two-Sided Pr > |Z|	1.0000

Z includes a continuity correction of 0.5.

Kruskal-Wallis Test

Chi-Square	0.0000
DF	1
Pr > Chi-Square	1.0000

Median Scores (Number of Points Above Median) for Variable WEIGHT
Classified by Variable Sex

Sex	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
F	2	1.0	1.0	0.577350	0.50
M	2	1.0	1.0	0.577350	0.50

Median Two-Sample Test

Statistic	1.0000
Z	0.0000
One-Sided Pr < Z	0.5000
Two-Sided Pr > |Z|	1.0000

Median One-Way Analysis

Chi-Square	0.0000
DF	1
Pr > Chi-Square	1.0000

NONPARAMETRIC COMPARISON OF FROG LENGTH BETWEEN SEXES FOR EACH EXPOSURE GROUP 722

-- ATRAZINE=10 --

The NPAR1WAY Procedure

Wilcoxon Scores (Rank Sums) for Variable WEIGHT
Classified by Variable Sex

Sex	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
F	2	5.0	5.0	1.290994	2.50
M	2	5.0	5.0	1.290994	2.50

Wilcoxon Two-Sample Test

Statistic	5.0000
Normal Approximation	
Z	0.0000
One-Sided Pr < Z	0.5000
Two-Sided Pr > |Z|	1.0000
t Approximation	
One-Sided Pr < Z	0.5000
Two-Sided Pr > |Z|	1.0000

Z includes a continuity correction of 0.5.

Kruskal-Wallis Test

Chi-Square	0.0000
DF	1
Pr > Chi-Square	1.0000

Median Scores (Number of Points Above Median) for Variable WEIGHT
Classified by Variable Sex

Sex	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
F	2	1.0	1.0	0.577350	0.50
M	2	1.0	1.0	0.577350	0.50

Median Two-Sample Test

Statistic	1.0000
Z	0.0000
One-Sided Pr < Z	0.5000
Two-Sided Pr > |Z|	1.0000

Median One-Way Analysis

Chi-Square	0.0000
DF	1
Pr > Chi-Square	1.0000

NONPARAMETRIC COMPARISON OF FROG LENGTH BETWEEN SEXES FOR EACH EXPOSURE GROUP 724

-- ATRAZINE=25 --

The NPAR1WAY Procedure

Wilcoxon Scores (Rank Sums) for Variable WEIGHT
Classified by Variable Sex

Sex	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
F	3	9.0	10.50	2.291288	3.0
M	3	12.0	10.50	2.291288	4.0

Wilcoxon Two-Sample Test

Statistic	9.0000
Normal Approximation	
Z	-0.4364
One-Sided Pr < Z	0.3313
Two-Sided Pr > |Z|	0.6625
t Approximation	
One-Sided Pr < Z	0.3404
Two-Sided Pr > |Z|	0.6807

Z includes a continuity correction of 0.5.

Kruskal-Wallis Test

Chi-Square	0.4286
DF	1
Pr > Chi-Square	0.5127

Median Scores (Number of Points Above Median) for Variable WEIGHT
Classified by Variable Sex

Sex	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
F	3	1.0	1.50	0.670820	0.333333
M	3	2.0	1.50	0.670820	0.666667

Median Two-Sample Test

Statistic	1.0000
Z	-0.7454
One-Sided Pr < Z	0.2280
Two-Sided Pr > |Z|	0.4561

Median One-Way Analysis

Chi-Square	0.5556
DF	1
Pr > Chi-Square	0.4561

Data Evaluation Report on South African Analytical Support– Hormone and Aromatase Analysis
EPA MRID Number 458675-01

Data Requirement::

EPA DP Barcode D288775

EPA MRID 458675-01
EPA Guideline 70-1(Special Study)

Test material: **Purity:** not reported
Common name Atrazine
Chemical name: IUPAC
CAS name 6-chloro-N-ethyl-N'-(1-methylethyl)-1,3,5-triazine-2,4-diamine
CAS No. 1912-24-9
Synonyms
EPA PC Code: 80803

Thomas M Steeger

Primary Reviewer: Thomas M. Steeger, Ph.D., Senior Biologist **Date:** April 1, 2003
Environmental Fate and Effects Division, ERB 4,
U. S. Environmental Protection Agency

Joseph C. Tietge

Secondary Reviewer(s): Joseph E. Tietge, M.S., Research Aquatic Biologist **Date:** April 16, 2003
Mid-Continent Ecology Division, National Health and Environmental Effects Research Laboratory (Duluth),
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Stephanie R Irene 4/29/03
Stephanie Irene, Ph.D., Senior Advisor **Date:**
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Mary Frankenberry
Mary J. Frankenberry, Senior Statistician **Date:** 4/28/03
Environmental Fate and Effects Division, ERB 3, U. S. Environmental Protection Agency

EPA PC Code 080803

Date Evaluation Completed: 06/01/2003

<u>**CITATION**</u>: Giesy, J. P., M. Hecker, and P. D. Jones. 2003. South African Analytical Support – Hormone and Aromatase Analysis (SA- 01C). Aquatic Toxicology Laboratory, Michigan State University, National Food Safety and Toxicology Center, E. Lansing, MI. Sponsor: Syngenta Crop Protection, Inc., Laboratory Study ID ECORISK Number MSU-07.

EXECUTIVE SUMMARY:

A field study was conducted in corn-growing (experimental) and non-corn growing (reference) areas in the Potchefstroom region of South Africa to determine whether exposure to atrazine and related triazines could affect aromatization of testosterone to estradiol in wild populations of African clawed frogs (*Xenopus laevis*) In this region, atrazine is typically applied in October to November. Most frogs were collected in April and May after the rainy season; however, in some atrazine-use areas, frog collections extended until mid-September due to the low sample sizes. Frogs were collected from baited traps after 48 hours, transferred to the lab, and housed individually in 2-L plastic cages for 48 hours before collecting samples. Blood samples were collected for plasma testosterone and estradiol analysis and gonad (one) samples were collected for aromatase activity analysis. Snout-vent length, body weight, and gonad weight were measured. Gonadosomatic index (GSI = gonad weight ÷ body weight), condition index (CI = body weight ÷ snout-vent length) and the ratio of plasma testosterone to estradiol were calculated.

Mean and median GSI were greater for both males and females in high atrazine exposure (experimental) sites compared to low atrazine exposure (reference) sites; however, there was no difference in body weight (CI) between high and low atrazine exposure sites. When comparisons were made between ponds with the highest atrazine concentrations to those with the lowest atrazine concentrations, males from high atrazine exposure sites had significantly lower median plasma testosterone than males from low atrazine exposure sites. Although male plasma testosterone was not significantly correlated with atrazine concentrations, there was a significant negative correlation between the log of male plasma testosterone levels and log of the atrazine degradate diaminochlorotriazine (DACT) concentration. Additionally, female plasma testosterone levels were negatively correlated with atrazine concentrations, and females collected from reference ponds had significantly higher testosterone levels than females collected from experimental sites.

Mean plasma estradiol concentrations were significantly lower in experimental site males and females compared to reference site animals. Male plasma estradiol was negatively correlated with log DACT concentrations, while female estradiol concentrations were negatively correlated with atrazine concentrations.

Ideally, a study should be designed based on the variability associated with the measurement endpoints. Sample sizes should reflect the number of test animals required to identify a specified difference within a given level of certainty. Potential sources of variability should also be identified and controlled to the extent possible. The current study did not appear to base its design on the variability associated with the range of measurement endpoints. Animals were collected over some fairly broad periods of time (up to 6 months) that would almost assure that animals within the same group would vary considerably in terms of development. Based on the number of animals collected, frogs appeared to be more abundant in reference sites than in corn-growing regions where half as many frogs were collected. The entire design was questionable, including extended sampling periods in ponds of questionable similarity, baiting with potentially hormone-laden liver/meat, and confinement in cages at unknown loading rates. These factors could confound the study's ability to reliably and reasonably quantify what "typical" steroid hormone levels and/or aromatase activity is for *Xenopus*. Additionally, the presence of atrazine and triazine degradates across all sites (reference and corn-growing) and the failure of the study to characterize the suite of pesticides other than triazines in the study sites contributes to the uncertain utility of this study in attempting to document potential effects of atrazine.

In order for such a study to be of value, the authors would need to conduct a pilot study first to establish the necessary sample size required to detect a specified difference in the biological attributes being measured within a certain level of confidence. The sample size would be based on the variability associated with a particular parameter. Unfortunately, the levels of variability associated with the parameters of interest in this study would likely require very high sample sizes. A more practical approach would be to identify major sources of variability and attempt to better control those conditions.

The current data suggest that atrazine and/or its degradates may be impacting plasma testosterone; however, the data are not conclusive and the mechanism underlying this phenomena can not be identified based on this study.

I. MATERIALS AND METHODS

GUIDELINE FOLLOWED: Nonguideline Study

COMPLIANCE: Not conducted under full GLP; however, most practices as defined by 40 CFR Part 160, August 19, 1989 were established for this study, including but not limited to:

- Written, authorized protocol
- Written, authorized Standard Operating Procedures for all key procedures.
- Organization and Personnel were sufficient in terms of number, education, training and experience.
- Facilities were of suitable size and construction
- Equipment used was of appropriate design and adequate capacity.
- Independent QA Inspections were conducted.
- Phase Report was written
- Raw data, documentation, records, protocols,.and final phase report will be archieved.

A. MATERIALS:

1. Test Material Atrazine

Description: Not reported

Lot No./Batch No. : Not reported

Purity: Not reported

Stability of compound under test conditions: Not reported

Storage conditions of test chemicals: Not reported

2. Test organism:

Species: African clawed frog (*Xenopus laevis*)

Age at test initiation: Adults
Weight at study initiation (mean and range): Not reported
Length at study initiation (mean and range): Not reported

Source: Adult *X. laevis* were field collected in two areas (non-corn growing and corn growing) in the vicinity of Potchefstrooom, South Africa, using traps baited with liver and meat scraps.

B. STUDY DESIGN:

Objective:

1. To determine whether exposure to atrazine and related triazines under field conditions in a major corn-growing area in South Africa could affect aromatization of testosterone and estradiol in wild populations of clawed frogs (*X. laevis*) and more specifically:

   a. to determine if exposure to triazines used in corn production affects plasma concentrations of the sex steroid hormones T and E2 in male and female *X. laevis* adults;
   b. to determine whether there are alterations in aromatase activity in adult *X. laevis* in both sexes in response to exposure to triazines;
   c. to evaluate the range of triazine concentrations, if any, that might lead to changes in plasma hormone concentrations and aromatase activity synthesis; and
   d. to evaluate the possible relevance of triazine-mediated alterations in the aromatization of T to E2 to reproduction.

1. Experimental Conditions

a) **Range-finding Study**:

b) **Definitive Study**

Table 1 . Experimental Parameters

Sampling was conducted in April and May approximately 6 months after atrazine was applied (October / November); water samples to characterize exposure was collected one month prior to frog sampling. Environmental parameters measured at each site included habitat description (vegetative cover and water depth). Water samples were analyzed for atrazine, related triazines, other pesticides and metals.

A total of eight sites in two adjacent regions (Viljoenskroon corn growing region = E for experimental were sampled; Potchefstroom non-corn growing region = C or R or control/reference) in South Africa.

Compounds of interest included atrazine, its metabolites desethylated atrazine (DEA), desisopropyl atrazine (DIA), diaminochlorotriazine (DACT), plus terbutylazine, simazine and acetochlor.

Non-corn growing sites had secchi disc readings ranging from 6.5 to 32 cm; pH ranged from 5.1 to 8.8; some of the control ponds were subject to drying (semi-permanent).

Corn-growing sites had surface areas ranging from 2,400 m^2 to 68,000 m^2; pH ranged from 7.2 - 10.8 and upper secchi disc value of 207 cm.

Frogs were sampled after the rainy season in April and May; however, at atrazine site E8, frogs were sampled over a six -month period (April - September) due to low sample sizes. Sampling at all other sites took place once or twice within a "few days." Frogs were collected in 10 baited traps and harvested after two days. Afterwards, animals were housed individually in 2-L plastic containers for 48 hours to recover from capture stress. Blood was collected by cardiac puncture; gonads were removed, weighed and measured. One gonad was fixed in 10% neutral buffered formalin or Bouin's, while the second was snap frozen. Gonadosomatic index (weight of the gonad ÷ snout-vent length) and the condition index (body weight ÷ snout-vent length) were calculated.

Concentrations of testosterone (T) and estradiol (E2) in blood plasma were measured by competitive ELISA as described by Cuisset *et al.* 1994 with modifications by Hecker *et al.* 2002 using COSTAR high binding plates. Working ranges of the assays were from 0.78 to 800 pg/well for both testosterone and estradiol.

Aromatase activity was measured following the protocol from Lephart and Simpson 1991 with minor modifications (Sanderson 2000). Less than 0.5 g of gonad was homogenized.

II. RESULTS and DISCUSSION: [All results discussed in this section and the next are those reported by the study authors. Although supplemental data are typically used in a qualitative manner only, EFED verified spreadsheet data and ran basic statistical analyses on the major study parameters. See attached appendix. If results appeared to differ in any substantive way, the difference was reported in the text below.]

Atrazine Concentrations

With the exception of site E8 (peak concentration in late January at 9 µg/l), the greatest concentrations of atrazine in pond water were observed at the end of the rainy season (March through May); highest atrazine concentrations were measured at sites E1 (4.1 µg/L), E6 (3.9 µg/L) and E8 (3.5 µg/L). Concentrations at C1, C3, C6 and E4 were low but could be quantified. The log concentrations of triazines DIA, DEA and atrazine were significantly correlated with pH of the water. ($p < 0.034$, r-range: 0.770 - 0.802). Log DIA also showed a positive linear regression with log temperature (7 = 0.801; p= 0.017) and log visual depth ® = 0.814; p = 0.014). Monitoring data (**Table 1**) indicated that measurable levels of atrazine were detected at all sampling sites (range 0.2 - 3.53 µg/L). The atrazine degradate desisopropyl atrazine (DIA) was as high in C3 (0.61 µg/L) as in many of the corn-growing sites, while diaminochlorotriazine (DACT) in C1 (3.91 µg/L) was the second highest concentration of the degradate detected at any of the sampling sites. Mean-measured atrazine concentrations, which were determined four weeks before frogs were collected and averaged over November 2001 to June 2002 (Table 2), indicated that atrazine in reference sites was generally an order of magnitude lower than sites located in corn-growing areas. However, all concentrations were apparently readily quantified.

Table 1. Concentrations of pesticides (µg/L) at the different sampling sites. Values were calculated from two individual samplings that were conducted with a time period in 2002 of 4 weeks before collecting *X. laevis*.

	C1	C3	C6	E1	E3	E4	E6	E8
Site	4/2	3/18	5/1	4/15	4/1	4/2	4/15	4/15
	4/15	4/2	5/13	5/1	4/15	4/15	5/1	5/1

Simazine	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	1.7	1.2
	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5		
DACT	3.91	<0.1	<0.1	<0.1	0.38	<0.1	5.45	1.24	
	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	
DIA	0.23	0.3	0.13	0.7	0.47	0.18	0.49	0.7	
	0.27	0.61	0.15	0.93	0.28	0.64	0.32	0.88	
DEA	0.18	0.15	0.12	1.29	0.43	0.11	1.09	1.04	
	<0.1	<0.1	0.13	1.16	0.38	<0.1	0.9	0.91	
Atrazine	0.28	0.36	<0.1	4.14	1.05	0.32	3.78	3.53	
	0.26	0.57	0.2	3.5	1.01	0.29	3.9	3.12	
Terbutyl	<0.1	<0.1	0.51	3.66	0.97	0.19	2.46	0.86	
	<0.1	<0.1	0.59	2.95	1.07	0.24	2.4	0.72	

Table 2. Mean concentrations of atrazine at the sampling locations measured during four weeks collecting frogs, and time weighted means of atrazine measured between November 2001 and June 2002. Values listed in ascending order of concentration

Location	Mean Atrazine Concentration 4 weeks Before Sampling	Location	Time-weighted Mean Atrazine Concentration
C6	0.13	C1	0.15
C1	0.27	C6	0.19
E4	0.31	C3	0.25
C3	0.47	E4	0.48
E3	1.0	E3	0.82
E8	3.3	E6	2.6
E1	3.8	E1	3.2
E6	3.8	E8	3.4

Gonadosomatic Index (GSI)

The means of the median GSI values for both female (t-test; $p = 0.0640$) and male (t-test; $p = 0.0725$) from corn growing areas were greater than the female and male means from the non-corn growing areas. The GSI of females form E1 and E8 were more variable than those at other locations.

Condition Index

No statistically significant differences were observed in the means of the site-specific CI values between the corn-growing and non-corn growing sites regardless of whether the analysis was based on means or medians.

Testosterone (T)

Plasma T concentrations were greater in males than in females except in reference pond C1 where mean male plasma testosterone (~105 pg/mL) was roughly an order of magnitude lower than females' mean plasma testosterone (~1005 pg/mL). At sites C6 and E6, males and females contained roughly similar amounts of plasma testosterone. For males, there was no significant difference between plasma T in frogs from corn-growing and non-corn growing sites, and there was no correlation between plasma T and atrazine. However, there was a significant negative correlation between the log of male plasma T concentration and the log of the DACT concentration ($r = -0.839$, $p = 0.009$) Median and mean plasma T concentrations of males were not significantly different between the experimental and reference sites. However, when the sites were grouped into either those with the four greatest and four least atrazine concentrations or into groups where atrazine concentrations were greater than 3 µg/L vs those with concentrations that were less than 1 µg/L, there were significant differences, with the frogs from the greater atrazine sites having less median concentrations of plasma T.

Although the report states that mean and median female plasma testosterone levels were significantly higher in experimental ponds than at reference sites (t-test, mean $p = 0.018$; median 0.0061), the figure (Figure 4) depicting this relationship suggests the opposite, *i.e.*, that median female plasma testosterone concentrations were higher in reference sites. This would better support the fact that female plasma T concentrations were negatively correlated with atrazine concentrations ($r = -0.725$; $p = 0.042$).

Estradiol (E2)

Median female plasma concentrations of E2 from experimental sites were significantly less than those of females from reference sites (t-test; $p = 0.0018$), but there was no significant difference for males. Mean plasma estradiol concentrations were less at experimental sites for both males (t-test, $p = 0.0186$) and females (t-test; $p = 0.0052$). Concentrations of E2 in the plasma of both males and females were more variable in reference sites than those from experimental sites. Male plasma E2 were not related to atrazine but were negatively correlated with the log of DACT concentrations ($r = -0.779$; $p = 0.023$). In females, plasma E2 was negatively correlated with atrazine concentration ($r = -0.833$; $p = 0.010$).

E2/T Ratio

Because of the magnitude of variability associated with this index, E2/T ratios for both males and females were not significantly different either between the experimental and reference sites or between other location groupings regardless of whether they were based on means or medians. E2/T ratio in males was negatively correlated with DIA concentrations ($r = -0.830$; $p = 0.011$). There were no significant relationships for E2/T ratio for females and any triazine residues.

Aromatase Activity

Aromatase levels in testes were not measurable at most sites. There were no significant differences in median aromatase activity in ovaries between experimental and reference or when locations were grouped based on the highest and lowest atrazine concentrations. The greatest variability in ovarian aromatase activity was at site E8 and was attributed to the extended sampling period (April to September). Log ovarian aromatase activity was correlated with the log of the 4WM water temperature (7 = 0.786; $p = 0.021$); aromatase activity was not correlated with atrazine concentration but was correlated with with the log of the DIA concentration ($r = 0.857$; $p = 0.007$).

There were statistically significant linear regressions between the logarithms of T and E2 in both males ($r = 0.757$; $p < 0.001$) and females ($r = 0.868$; $p < 0.001$). Both sexes also exhibited positive relationships between log E2 and log E2/T (males: $r = 0.804$, $p < 0.001$; females: $r = 0.582$, $p < 0.001$). However, aromatase in females was not related to sex steroid concentrations.

C. <u>REPORTED STATISTICS</u>: Data used in statistical tests were stratified by sex, *i.e.*, male and female data analyzed separately. Studies were designed to be analyzed by both fixed-effects models and by regression type statistics because specific locations could not be classified as exposed or unexposed since atrazine residues were also detected in non-corn growing "reference sites". Statistical comparisons were made between the median concentrations of plasma testosterone and estradiol as well as the E2/T ratio, the GSI, the EI and gonadal aromatase; regression analysis between atrazine concentration and these parameters was also conducted.. For statistical tests, locations were grouped several ways: grouped based on whether they were in corn-growing (E) or non-corn growing (C) areas; grouped with a mean atrazine concentration ≤ 1.0 µg/L (C1, C3, C6, E3, E4) versus > 3 µg/L (E1, E6, E8). Finally, study sites were grouped with the four sites with the 4 smallest means in one group, and the remaining four sites with the four largest means in the other group. Two-sided two sample t-test was used to compare the mean of the site values in the first group to the mean of the site values in the second group. Two-sample Kolmogorov-Smirnov test was used to compare individual locations. Linear model comparisons of four -week average atrazine and/or metabolites were used for each parameter.

D. VERIFICATION OF STATISTICAL RESULTS: See attached SAS© output (Statistical Analysis System, Release 8.01, Cary, North Carolina.

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E. STUDY DEFICIENCIES:

Atrazine and/or triazine degradates were present in control sites.

While the low sample size at sites E1 and E8 may have been explained by the sudden introduction of predatory catfish, it does not explain why the remaining corn-growing sites (E3, E4 and E6) also yielded fewer frogs than non-corn growing sites.

Because frogs were sampled over disproportionate periods of time, e.g., 6 months for site E8, samples would likely contain animals at different stages of development

Frog traps were baited with liver/meat scraps that may have contained hormones.

Following capture, frogs were housed individually in "clean water" for 48 hours. The volume and characteristics of this water were not described in the study.

F. REVIEWER'S COMMENTS:

The large amount of variability associated with aromatase and plasma hormone data in laboratory studies make it difficult to understand the utility of conducting a field study with these parameters. Collecting animals over a six-month period would also contribute to extremely high variability.

Atrazine is typically applied in October / November when corn is planted in South Africa. Frog sampling was conducted after the rainy season in April to May and water samples to occurred within a time span of four weeks before frogs were sampled. At exposure site E8, frogs were sampled over a 6-month time frame. The fewer number of frogs was attributed to a high runoff event that washed catfish into the pond; the catfish preyed on the frogs.

Reference and atrazine-exposure sites appeared to be widely divergent, ranging from 5.1 - 8.8 at reference sites and 7.2 - 10.8 at atrazine sites.

Frogs were attracted into baited traps containing liver and/or scrap meat which may have contained hormones. Apparently trapped animals were held in cages for two days which should have provided sufficient time to consume all of the bait. Also, depending on catch rates, the loading within the cages could have stressed the frogs. Although it doesn't state the desired sample size in the report's methodology, apparently (based on the raw data), 20 male and 20 female frogs were to be sampled at each site. **Table 3** shows that while the desired sample size was generally available for frogs collected in non-corn growing areas, sample sizes were minimally 50% lower in corn-growing areas. In site E1, sampling for female frogs extended into late June, and at site E8 sampling extended into mid-August for females and into mid-September for males. Extended sampling periods at these two sites may have resulted in collecting animals that were at considerably different states of development. The gonadosomatic index and ovarian aromatase activity in females collected from sites E1 and E8 expressed the highest variability relative to any of the other treatment sites.

Table 3. Summary of total number of *Xenopus laevis* collected from April through May 2002 at non-corn growing (C) and corn growing (E) sites in South Africa.

Site	Males	Females
C1	20 (Apr 21 - Apr 23)	20 (Apr 21 - May 1)
C3	17 (Apr 4 - Apr 11)	20 (Apr 4 - Apr 11)
C6	20 (May 13 - May 15)	20 (May 13 - May 14)
E1	8 (April 29 - May 7)	8 (April 29 - June 29)
E3	10 (Apr 9 - Apr 17)	10 (Apr 9 - Apr 18)
E4	10 (Apr 13 - Apr 15)	10 (Apr 14 - Apr 16)
E6	10 (May 5 - May 9)	10 (May 5 - May 6)
E8	6 (Apr 29 - Sep 17)	15 (Apr 29 - Aug 17)

Statistical analyses were more regression-based because specific sampling locations could not be classified as exposed or unexposed. There was a range of concentrations of primary residues of concern, with greater concentrations in the corn-growing areas; however, residues were also detected in non-corn growing areas. In Phase II of the study (Smith *et al.* 2003), the authors suggested that atrazine residues in reference ponds may be a result of atmospheric deposition (wind).

The GSI and aromatase ovarian activity of females from E8 were more variable than those at other locations. This variability may have resulted from the long sampling period (six months) and the considerable changes in the frogs' reproductive state.

Gonadosomatic index suggests that both males and females collected in corn-growing areas were more sexually developed than their counterparts in "reference" sites.

Comparisons between male and female plasma testosterone levels were also are hindered by the considerable variability within pond and within treatment groups. Although median plasma testosterone levels were qualitatively different. Males in reference ponds had higher median plasma testosterone levels than males in experimental ponds by approximately a factor of 2. Similarly, females in reference ponds had higher median plasma testosterone levels than females in experimental ponds by approximately a factor of 8. However, given the magnitude of variability, it wasn't possible to document statistical differences in means or medians. Only when the samples were grouped based on the three highest and three lowest atrazine exposures could statistical differences be documented in median T levels. Also, the text may be in error when it concludes that female plasma testosterone levels were higher in corn-growing areas (experimental) than in non-corn growing areas because the statement appears to contradict the figure (Figure 4) depicting the data and from the significant correlation showing that female plasma testosterone is negatively related to atrazine.

Based on Figure 5, median plasma E2 concentrations in males from reference sites were approximately similar to female plasma estradiol concentrations in reference sites, while median male plasma estradiol at reference sites was approximately five-fold higher than plasma estradiol concentrations of males from experimental sites. A similar pattern existed for females where reference site animals had roughly six-fold higher estradiol concentrations than females at experimental sites.

Although the study authors conclude that there were no statistical differences in the ratio of estradiol to testosterone (E2/T), in a qualitative sense, the median values for females were relatively consistent across experimental and reference sites. However, the median ratio for males tended to be roughly double in animals collected from reference sites compared to those collected at experimental sites.

Although not considered very predictive (correlation coefficients ranged from 0.22 to 0.38), there were significant correlations ($p<0.026$) between condition index and both gonadosomatic index and log aromatase activity in females. This correlation is intuitively obvious because more gravid females should weigh more and have higher aromatase activity than less developed animals.

The report discussed a number of statistical differences that were detected and how misleading this could be based on the number of statistical comparisons that were run. The authors suggested that if Bonferroni's correction had been made, none of the p values would have been significant. The Bonferroni correction is used to control experiment error rates and is obtained by dividing the overall α-level for the study by the number of groups being compared. If the study has a large number of means, however, this process can be very conservative. It may also fail to detect significant differences between groups and increase the likelihood of making a Type II error.

In this study, the authors discuss the relevancy of the Tavera-Mendoza *et al.* (2002*a* and 2002*b*) studies which showed decreased testicular volume in animals exposed to 21 μg/L and how the current study demonstrated increases in GSI. At the same time, though, they note that the current study's maximum exposure values were roughly five-fold lower than those used in the Tavera-Mendoza study and that improved food sources may have resulted in increased gonadal weight. Others differences in the Tavera-Mendoza study include: the laboratory study was presumably conducted on animals that were probably in the same state of development, the environmental conditions in the laboratory were better defined, and the laboratory study was an acute exposure (48 hour) as opposed to the current chronic study.

The authors state that there is no plausible mechanism that could be postulated to explain the negative correlation between plasma E2 concentrations in females and concentrations of atrazine in pond water; however, they note that correlations do not imply causality.

Since there were no differences in aromatase activities between locations or significant correlations between exposure to atrazine and aromatase activity, the authors believe that these findings do not support the Hayes *et al.* 2002*a, b* hypothesis that atrazine causes an increase in the production of estrogen by inducing aromatase activity. The fact that plasma T concentrations were less in groups with the higher atrazine exposure values was viewed as being consistent with the hypothesis that atrazine decreased plasma T, but the fact that atrazine concentrations were not correlated with plasma T was viewed as suggesting that the statistical significance was artifactual. The significant negative correlation between the E2/T ratio of males and DIA was viewed as being inconsistent with Sanderson *et al.* 2001, showing that DIA significantly increased CYP-19 mRNA expression as well as aromatase activity. They concluded, though, that the differences in plasma steroid hormone concentrations were not caused by up-regulation of aromatase activity. The report states "although significant negative correlations were observed between exposure to atrazine and its metabolite DACT and plasma concentrations of sex steroids in wild *X. laevis*, it is impossible from this study to conclude the exact cause or accuracy of these differences." The experimental corn-growing regions were subject to a range of chemicals there were not fully characterized and that may have contributed to the observed statistical differences.

G. CONCLUSIONS:

This study indicates that plasma hormone levels and gonadal aromatase activity are highly variable and depend on a number of factors that were not anticipated when the study was designed. Reference ponds in areas of South Africa where corn is not grown were subject to variable levels of atrazine contamination that approached exposure values for ponds in corn-growing regions. Collecting frogs over protracted periods of time may have constituted one of the greatest sources of variability because the frogs were probably at different stages of their sexual cycles. It is unclear what effect the bait used to attract frogs into traps may have had on hormone levels. In spite of the fact that in some comparisons the study was unable to differentiate male and female hormone levels, some evidence suggested that males subjected to higher atrazine concentrations tended to have reduced plasma testosterone and that higher concentrations of the atrazine degradate DACT were associated with reduced plasma testosterone levels. Also, contrary to what the authors state, median female plasma testosterone appeared to be significantly higher in reference sites where atrazine exposure was reduced. This conclusion is consistent with the authors' observation that female plasma testosterone was negatively correlated with atrazine concentration. Mean plasma estradiol concentrations in both males and females were significantly lower in high atrazine exposure sites, and like testosterone, male estradiol concentrations were negatively correlated with concentrations of the atrazine degradate DACT. Female estradiol concentrations were negatively correlated with atrazine concentrations. Gonadal aromatase activity in males could not be accurately characterized because of its low level across the majority of collection sites, while high variability in female aromatase activity made all the comparisons insignificant. Aromatase activity, though, was negatively correlated with concentrations of the atrazine degradate DIA.

Ideally, a study should be designed based on the variability associated with the measurement endpoints. Sample sizes should reflect the number of test animals required to identify a specified difference within a given level of certainty. Potential sources of variability should also be identified and controlled to the extent possible. The current study did not appear to base its design on the variability associated with the range of measurement endpoints. Animals were collected over broad periods of time (up to six months) which would assure variation within the same group in terms of development. Based on the number of animals collected, frogs appeared to be more abundant in reference sites than in corn-growing regions where half as many frogs were collected. The entire design was questionable, including: the extended sampling periods in ponds of questionable similarity, baiting with potentially hormone-laden liver/meat, and confinement in cages at unknown loading rates. These factors could confound the study's ability to reliably and reasonably quantify "typical" steroid hormone levels and/or aromatase activity in *Xenopus*. Additionally, the presence of atrazine and triazine degradates across all sites (reference and corn-growing) and the failure to characterize other pesticides at the study sites limits the usefulness of this study in determining potential effects of atrazine and in supporting the study's hypothesis.

Before a field study of this scope is undertaken, researchers need to conduct a pilot study to establish the necessary sample size for detecting a specified difference in the biological attribute being measured within a certain level of confidence. The sample size would be based on the variability associated with a particular parameter. Unfortunately, the levels of variability associated with the parameters of interest in this study would likely require very high sample sizes. A more practical approach would be to identify major sources of variability and then try to control those conditions.

The current data suggest that atrazine and/or its degradates may be impacting plasma testosterone; however, the data are not conclusive for the reasons cited above.

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MEAN LENGTHS OF FROGS COLLECTED BY POND AND SEX 391

Obs	POND	Sex	GROUP	_TYPE_	_FREQ_	LENGTH	L_SD	L_CV
1	C1	F	REF	0	20	69.5450	8.0156	11.5258
2	C1	M	REF	0	20	58.0000	6.6373	11.4436
3	C3	F	REF	0	20	68.9100	14.4936	21.0326
4	C3	M	REF	0	20	56.9882	10.9170	19.1565
5	C6	F	REF	0	20	76.9500	7.0433	9.1531
6	C6	M	REF	0	20	72.1100	7.1908	9.9721
7	E1	F	EXP	0	10	64.4833	14.3272	22.2185
8	E1	M	EXP	0	10	59.2000	9.4870	16.0253
9	E3	F	EXP	0	10	74.3900	6.9437	9.3341
10	E3	M	EXP	0	10	66.6300	6.9863	10.4853
11	E4	F	EXP	0	10	77.5400	10.9218	14.0853
12	E4	M	EXP	0	10	63.8300	3.3549	5.2561
13	E6	F	EXP	0	10	88.5000	9.5274	10.7654
14	E6	M	EXP	0	10	62.6600	9.5755	15.2816
15	E8	F	EXP	0	15	64.4733	13.8195	21.4344
16	E8	M	EXP	0	10	60.7167	7.2841	11.9968

ANOVA FOR LENGTH OF FROGS BETWEEN PONDS BY SEX 392

-- Sex=F --

The ANOVA Procedure

Class Level Information

Class	Levels	Values
POND	8	C1 C3 C6 E1 E3 E4 E6 E8

Number of observations 115

NOTE: Due to missing values, only 111 observations can be used in this analysis.

Dependent Variable: LENGTH

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	7	5026.98373	718.14053	6.07	<.0001
Error	103	12178.98817	118.24260		
Corrected Total	110	17205.97189			

R-Square	Coeff Var	Root MSE	LENGTH Mean
0.292165	14.96340	10.87394	72.67027

Source	DF	Anova SS	Mean Square	F Value	Pr > F
POND	7	5026.983725	718.140532	6.07	<.0001

Levene's Test for Homogeneity of LENGTH Variance
ANOVA of Squared Deviations from Group Means

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
POND	7	432098	61728.3	3.51	0.0020
Error	103	1810469	17577.4		

Bartlett's Test for Homogeneity of LENGTH Variance

Source	DF	Chi-Square	Pr > ChiSq
POND	7	17.4523	0.0147

Dunnett's t Tests for LENGTH

NOTE: This test controls the Type I experimentwise error for comparisons of all treatments against a control.

Alpha	0.05

Error Degrees of Freedom	103
Error Mean Square	118.2426
Critical Value of Dunnett's t	2.69261

Comparisons significant at the 0.05 level are indicated by ***.

POND Comparison	Difference Between Means	Simultaneous 95% Confidence Limits		
E6 - C1	18.955	7.615	30.295	***
E4 - C1	7.995	-3.345	19.335	
C6 - C1	7.405	-1.854	16.664	
E3 - C1	4.845	-6.495	16.185	
C3 - C1	-0.635	-9.894	8.624	
E1 - C1	-5.062	-18.690	8.567	
E8 - C1	-5.072	-15.072	4.929	

ANOVA FOR LENGTH OF FROGS BETWEEN PONDS BY SEX 396

-- Sex=M --

The ANOVA Procedure

Class Level Information

Class	Levels	Values
POND	8	C1 C3 C6 E1 E3 E4 E6 E8

Number of observations 110

NOTE: Due to missing values, only 101 observations can be used in this analysis.

Dependent Variable: LENGTH

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	7	3055.667841	436.523977	6.78	<.0001
Error	93	5987.449980	64.381183		
Corrected Total	100	9043.117822			

R-Square	Coeff Var	Root MSE	LENGTH Mean
0.337900	12.78217	8.023789	62.77327

Source	DF	Anova SS	Mean Square	F Value	Pr > F
POND	7	3055.667841	436.523977	6.78	<.0001

Levene's Test for Homogeneity of LENGTH Variance
ANOVA of Squared Deviations from Group Means

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
POND	7	92002.7	13143.2	2.03	0.0591
Error	93	601693	6469.8		

Bartlett's Test for Homogeneity of LENGTH Variance

Source	DF	Chi-Square	Pr > ChiSq
POND	7	14.5701	0.0419

Dunnett's t Tests for LENGTH

NOTE: This test controls the Type I experimentwise error for comparisons of all treatments against a control.

Alpha	0.05
Error Degrees of Freedom	93
Error Mean Square	64.38118
Critical Value of Dunnett's t	2.70384

Comparisons significant at the 0.05 level are indicated by ***.

POND Comparison	Difference Between Means	Simultaneous 95% Confidence Limits		
C6 - C1	14.110	7.249	20.971	***
E3 - C1	8.630	0.228	17.032	***
E4 - C1	5.830	-2.572	14.232	
E6 - C1	4.660	-3.742	13.062	
E8 - C1	2.717	-7.382	12.815	
E1 - C1	1.200	-7.876	10.276	
C3 - C1	-1.012	-8.169	6.145	

NONPARAMETRIC COMPARISON OF FROG LENGTH BETWEEN PONDS 400

--- Sex=F ---

The NPAR1WAY Procedure

Wilcoxon Scores (Rank Sums) for Variable LENGTH
Classified by Variable POND

POND	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
C1	20	896.00	1120.0	130.323757	44.800000
C3	20	975.50	1120.0	130.323757	48.775000
C6	20	1361.00	1120.0	130.323757	68.050000
E1	6	209.00	336.0	76.675775	34.833333
E3	10	593.00	560.0	97.084207	59.300000
E4	10	656.00	560.0	97.084207	65.600000
E6	10	945.50	560.0	97.084207	94.550000
E8	15	580.00	840.0	115.922875	38.666667

Average scores were used for ties.

Kruskal-Wallis Test

Chi-Square	28.5206
DF	7
Pr > Chi-Square	0.0002

The NPAR1WAY Procedure

Median Scores (Number of Points Above Median) for Variable LENGTH
Classified by Variable POND

POND	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
C1	20	7.0	9.909910	2.033723	0.350000
C3	20	6.0	9.909910	2.033723	0.300000
C6	20	14.0	9.909910	2.033723	0.700000
E1	6	1.0	2.972973	1.196537	0.166667
E3	10	6.0	4.954955	1.515014	0.600000
E4	10	5.0	4.954955	1.515014	0.500000
E6	10	10.0	4.954955	1.515014	1.000000
E8	15	6.0	7.432432	1.808995	0.400000

Average scores were used for ties.

Median One-Way Analysis

Chi-Square	21.6625
DF	7
Pr > Chi-Square	0.0029

-- Sex=M --

Wilcoxon Scores (Rank Sums) for Variable LENGTH
Classified by Variable POND

POND	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
C1	20	713.00	1020.0	117.339834	35.650000
C3	17	559.00	867.0	110.167138	32.882353
C6	20	1600.50	1020.0	117.339834	80.025000
E1	8	312.50	408.0	79.519644	39.062500
E3	10	644.50	510.0	87.944495	64.450000
E4	10	552.50	510.0	87.944495	55.250000
E6	10	494.00	510.0	87.944495	49.400000
E8	6	275.00	306.0	69.602588	45.833333

Average scores were used for ties.

Kruskal-Wallis Test

Chi-Square	35.4807
DF	7
Pr > Chi-Square	<.0001

The NPAR1WAY Procedure

Median Scores (Number of Points Above Median) for Variable LENGTH
Classified by Variable POND

POND	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
C1	20	6.0	9.900990	2.012363	0.300000
C3	17	3.0	8.415842	1.889352	0.176471
C6	20	20.0	9.900990	2.012363	1.000000
E1	8	2.0	3.960396	1.363751	0.250000
E3	10	6.0	4.950495	1.508236	0.600000
E4	10	6.0	4.950495	1.508236	0.600000
E6	10	4.0	4.950495	1.508236	0.400000
E8	6	3.0	2.970297	1.193675	0.500000

Average scores were used for ties.

Median One-Way Analysis

Chi-Square	33.1792
DF	7
Pr > Chi-Square	<.0001

ANOVA FOR LENGTH OF FROGS BETWEEN REFERENCE (REF) AND EXPERIMENTAL (EXP) SITES 404

-- Sex=F --

The ANOVA Procedure

Class Level Information

Class	Levels	Values
GROUP	2	EXP REF

Number of observations 8

Dependent Variable: LENGTH

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	1	8.0782352	8.0782352	0.11	0.7524
Error	6	444.1424033	74.0237339		
Corrected Total	7	452.2206385			

R-Square	Coeff Var	Root MSE	LENGTH Mean
0.017863	11.76994	8.603705	73.09896

Source	DF	Anova SS	Mean Square	F Value	Pr > F
GROUP	1	8.07823521	8.07823521	0.11	0.7524

Levene's Test for Homogeneity of LENGTH Variance
ANOVA of Squared Deviations from Group Means

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
GROUP	1	8547.2	8547.2	1.76	0.2326
Error	6	29101.8	4850.3		

Bartlett's Test for Homogeneity of LENGTH Variance

Source	DF	Chi-Square	Pr > ChiSq
GROUP	1	1.1508	0.2834

Dunnett's t Tests for LENGTH

NOTE: This test controls the Type I experimentwise error for comparisons of all treatments against a control.

Alpha	0.05
Error Degrees of Freedom	6
Error Mean Square	74.02373
Critical Value of Dunnett's t	2.44695

Comparisons significant at the 0.05 level are indicated by ***.

GROUP Comparison	Difference Between Means	Simultaneous 95% Confidence Limits	
REF - EXP	-2.076	-17.451	13.299

ANOVA FOR LENGTH OF FROGS BETWEEN REFERENCE (REF) AND EXPERIMENTAL (EXP) SITES 408

-- Sex=M --

Class Level Information

Class	Levels	Values
GROUP	2	EXP REF

Number of observations 8

Dependent Variable: LENGTH

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	1	0.1091324	0.1091324	0.00	0.9533
Error	6	175.7919208	29.2986535		
Corrected Total	7	175.9010531			

R-Square	Coeff Var	Root MSE	LENGTH Mean
0.000620	8.658181	5.412823	62.51686

Source	DF	Anova SS	Mean Square	F Value	Pr > F
GROUP	1	0.10913236	0.10913236	0.00	0.9533

Levene's Test for Homogeneity of LENGTH Variance
ANOVA of Squared Deviations from Group Means

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
GROUP	1	3162.6	3162.6	5.27	0.0615
Error	6	3600.4	600.1		

Bartlett's Test for Homogeneity of LENGTH Variance

Source	DF	Chi-Square	Pr > ChiSq
GROUP	1	2.7649	0.0964

Dunnett's t Tests for LENGTH

NOTE: This test controls the Type I experimentwise error for comparisons of all treatments against a control.

Alpha	0.05
Error Degrees of Freedom	6
Error Mean Square	29.29865
Critical Value of Dunnett's t	2.44695

Comparisons significant at the 0.05 level are indicated by ***.

GROUP Comparison	Difference Between Means	Simultaneous 95% Confidence Limits	
REF - EXP	-0.2413	-9.9140	9.4315

NONPARAMETRIC COMPARISON OF FROG LENGTH BETWEEN REFERENCE AND EXPERIMENTAL SITES 412

-- Sex=F --

The NPAR1WAY Procedure

Wilcoxon Scores (Rank Sums) for Variable LENGTH
Classified by Variable GROUP

GROUP	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
EXP	5	23.0	22.50	3.354102	4.600000
REF	3	13.0	13.50	3.354102	4.333333

Wilcoxon Two-Sample Test

Statistic	13.0000
Normal Approximation	
Z	0.0000
One-Sided Pr < Z	0.5000
Two-Sided Pr > |Z|	1.0000
t Approximation	
One-Sided Pr < Z	0.5000
Two-Sided Pr > |Z|	1.0000

Z includes a continuity correction of 0.5.

Kruskal-Wallis Test

Chi-Square	0.0222
DF	1
Pr > Chi-Square	0.8815

Median Scores (Number of Points Above Median) for Variable LENGTH
Classified by Variable GROUP

GROUP	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
EXP	5	3.0	2.50	0.731925	0.600000
REF	3	1.0	1.50	0.731925	0.333333

Median Two-Sample Test

Statistic	1.0000
Z	-0.6831
One-Sided Pr < Z	0.2473
Two-Sided Pr > |Z|	0.4945

Median One-Way Analysis

Chi-Square	0.4667
DF	1
Pr > Chi-Square	0.4945

NONPARAMETRIC COMPARISON OF FROG LENGTH BETWEEN REFERENCE AND EXPERIMENTAL SITES 414

--- Sex=M ---

The NPAR1WAY Procedure

Wilcoxon Scores (Rank Sums) for Variable LENGTH
Classified by Variable GROUP

GROUP	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
EXP	5	25.0	22.50	3.354102	5.000000
REF	3	11.0	13.50	3.354102	3.666667

Wilcoxon Two-Sample Test

Statistic	11.0000
Normal Approximation	
Z	-0.5963
One-Sided Pr < Z	0.2755
Two-Sided Pr > |Z|	0.5510
t Approximation	
One-Sided Pr < Z	0.2849
Two-Sided Pr > |Z|	0.5698

Z includes a continuity correction of 0.5.

Kruskal-Wallis Test

Chi-Square	0.5556
DF	1
Pr > Chi-Square	0.4561

The NPAR1WAY Procedure

Median Scores (Number of Points Above Median) for Variable LENGTH
Classified by Variable GROUP

GROUP	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
EXP	5	3.0	2.50	0.731925	0.600000
REF	3	1.0	1.50	0.731925	0.333333

Median Two-Sample Test

Statistic	1.0000
Z	-0.6831
One-Sided Pr < Z	0.2473
Two-Sided Pr > |Z|	0.4945

Median One-Way Analysis

Chi-Square	0.4667
DF	1
Pr > Chi-Square	0.4945

MEAN WEIGHTS OF FROGS COLLECTED BY POND AND SEX 416

Obs	POND	Sex	GROUP	_TYPE_	_FREQ_	WEIGHT	W_SD	W_CV
1	C1	F	REF	0	20	32.8315	11.1449	33.9459
2	C1	M	REF	0	20	19.4445	6.1022	31.3826
3	C3	F	REF	0	20	35.3455	20.2334	57.2446
4	C3	M	REF	0	20	20.7541	13.2996	64.0818
5	C6	F	REF	0	20	41.2495	10.4261	25.2757
6	C6	M	REF	0	20	37.2590	12.0340	32.2982
7	E1	F	EXP	0	10	29.1450	19.2315	65.9856
8	E1	M	EXP	0	10	23.3888	14.4006	61.5704
9	E3	F	EXP	0	10	39.3810	8.4697	21.5071
10	E3	M	EXP	0	10	32.5740	8.9938	27.6105
11	E4	F	EXP	0	10	48.8210	18.5235	37.9417
12	E4	M	EXP	0	10	29.9000	4.1673	13.9376
13	E6	F	EXP	0	10	66.9560	22.6873	33.8838
14	E6	M	EXP	0	10	26.1050	12.3935	47.4758
15	E8	F	EXP	0	15	29.8047	18.8819	63.3521
16	E8	M	EXP	0	10	23.0917	8.7925	38.0765

-- Sex=F --

The ANOVA Procedure

Class Level Information

Class	Levels	Values
POND	8	C1 C3 C6 E1 E3 E4 E6 E8

Number of observations 115

NOTE: Due to missing values, only 111 observations can be used in this analysis.
Dependent Variable: WEIGHT

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	7	11751.86955	1678.83851	6.31	<.0001
Error	103	27410.49679	266.12133		
Corrected Total	110	39162.36634			

R-Square	Coeff Var	Root MSE	WEIGHT Mean
0.300081	41.51186	16.31323	39.29775

Source	DF	Anova SS	Mean Square	F Value	Pr > F
POND	7	11751.86955	1678.83851	6.31	<.0001

Levene's Test for Homogeneity of WEIGHT Variance
ANOVA of Squared Deviations from Group Means

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
POND	7	2120258	302894	2.53	0.0190
Error	103	12311095	119525		

Bartlett's Test for Homogeneity of WEIGHT Variance

Source	DF	Chi-Square	Pr > ChiSq
POND	7	20.4080	0.0048

Dunnett's t Tests for WEIGHT

NOTE: This test controls the Type I experimentwise error for comparisons of all treatments against a control.

Alpha	0.05
Error Degrees of Freedom	103
Error Mean Square	266.1213
Critical Value of Dunnett's t	2.69261

Comparisons significant at the 0.05 level are indicated by ***.

POND Comparison	Difference Between Means	Simultaneous 95% Confidence Limits		
E6 - C1	34.124	17.112	51.137	***
E4 - C1	15.990	-1.023	33.002	
C6 - C1	8.418	-5.472	22.308	
E3 - C1	6.549	-10.463	23.562	
C3 - C1	2.514	-11.376	16.404	
E8 - C1	-3.027	-18.030	11.976	
E1 - C1	-3.687	-24.133	16.760	

ANOVA FOR WEIGHT OF FROGS BETWEEN PONDS BY SEX 421

-- Sex=M --

The ANOVA Procedure

Class Level Information

Class	Levels	Values
POND	8	C1 C3 C6 E1 E3 E4 E6 E8

Number of observations 110

NOTE: Due to missing values, only 101 observations can be used in this analysis.

Dependent Variable: WEIGHT

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	7	4500.49282	642.92755	5.75	<.0001
Error	93	10393.96375	111.76305		
Corrected Total	100	14894.45657			

R-Square	Coeff Var	Root MSE	WEIGHT Mean
0.302159	39.57071	10.57180	26.71624

Source	DF	Anova SS	Mean Square	F Value	Pr > F
POND	7	4500.492823	642.927546	5.75	<.0001

Levene's Test for Homogeneity of WEIGHT Variance
ANOVA of Squared Deviations from Group Means

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
POND	7	339909	48558.4	1.40	0.2144
Error	93	3223997	34666.6		

Bartlett's Test for Homogeneity of WEIGHT Variance

Source	DF	Chi-Square	Pr > ChiSq
POND	7	22.0595	0.0025

Dunnett's t Tests for WEIGHT

NOTE: This test controls the Type I experimentwise error for comparisons of all treatments against a control.

Alpha	0.05
Error Degrees of Freedom	93
Error Mean Square	111.7631
Critical Value of Dunnett's t	2.70384

Comparisons significant at the 0.05 level are indicated by ***.

POND Comparison	Difference Between Means	Simultaneous 95% Confidence Limits		
C6 - C1	17.815	8.775	26.854	***
E3 - C1	13.130	2.059	24.200	***
E4 - C1	10.455	-0.615	21.526	
E6 - C1	6.661	-4.410	17.731	
E1 - C1	3.944	-8.013	15.902	
E8 - C1	3.647	-9.658	16.953	
C3 - C1	1.310	-8.120	10.739	

-- Sex=F --

The NPAR1WAY Procedure

Wilcoxon Scores (Rank Sums) for Variable WEIGHT
Classified by Variable POND

POND	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
C1	20	895.50	1120.0	130.332621	44.775000
C3	20	971.00	1120.0	130.332621	48.550000
C6	20	1266.00	1120.0	130.332621	63.300000
E1	6	215.50	336.0	76.680990	35.916667
E3	10	601.00	560.0	97.090810	60.100000
E4	10	724.00	560.0	97.090810	72.400000
E6	10	950.00	560.0	97.090810	95.000000
E8	15	593.00	840.0	115.930760	39.533333

Average scores were used for ties.

Kruskal-Wallis Test

Chi-Square	28.2346
DF	7
Pr > Chi-Square	0.0002

Median Scores (Number of Points Above Median) for Variable WEIGHT
Classified by Variable POND

POND	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
C1	20	7.0	9.909910	2.033723	0.350000
C3	20	8.0	9.909910	2.033723	0.400000
C6	20	12.0	9.909910	2.033723	0.600000
E1	6	1.0	2.972973	1.196537	0.166667
E3	10	6.0	4.954955	1.515014	0.600000
E4	10	6.0	4.954955	1.515014	0.600000
E6	10	10.0	4.954955	1.515014	1.000000
E8	15	5.0	7.432432	1.808995	0.333333

Average scores were used for ties.

Median One-Way Analysis

Chi-Square	18.3589
DF	7
Pr > Chi-Square	0.0105

NONPARAMETRIC COMPARISON OF FROG WEIGHT BETWEEN PONDS 427

-- Sex=M --

The NPAR1WAY Procedure

Wilcoxon Scores (Rank Sums) for Variable WEIGHT
Classified by Variable POND

POND	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
C1	20	649.0	1020.0	117.344960	32.450000
C3	17	568.0	867.0	110.171950	33.411765
C6	20	1525.0	1020.0	117.344960	76.250000
E1	8	311.0	408.0	79.523118	38.875000
E3	10	681.0	510.0	87.948337	68.100000
E4	10	658.0	510.0	87.948337	65.800000
E6	10	484.0	510.0	87.948337	48.400000
E8	6	275.0	306.0	69.605629	45.833333

Average scores were used for ties.

Kruskal-Wallis Test

Chi-Square	36.5882
DF	7
Pr > Chi-Square	<.0001

Median Scores (Number of Points Above Median) for Variable WEIGHT
Classified by Variable POND

POND	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
C1	20	4.0	9.900990	2.012363	0.200000
C3	17	4.0	8.415842	1.889352	0.235294
C6	20	18.0	9.900990	2.012363	0.900000
E1	8	2.0	3.960396	1.363751	0.250000
E3	10	6.0	4.950495	1.508236	0.600000
E4	10	9.0	4.950495	1.508236	0.900000
E6	10	4.0	4.950495	1.508236	0.400000
E8	6	3.0	2.970297	1.193675	0.500000

Average scores were used for ties.

Median One-Way Analysis

Chi-Square	33.6219
DF	7
Pr > Chi-Square	<.0001

ANOVA FOR WEIGHT OF FROGS BETWEEN REFERENCE (REF) AND EXPERIMENTAL (EXP) SITES 429

-- Sex=F --

The ANOVA Procedure

Class Level Information

Class	Levels	Values
GROUP	2	EXP REF

Number of observations 8

Dependent Variable: WEIGHT

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	1	75.510261	75.510261	0.44	0.5307
Error	6	1024.136445	170.689408		
Corrected Total	7	1099.646706			

R-Square	Coeff Var	Root MSE	WEIGHT Mean
0.068668	32.30525	13.06482	40.44177

Source	DF	Anova SS	Mean Square	F Value	Pr > F
GROUP	1	75.51026075	75.51026075	0.44	0.5307

Levene's Test for Homogeneity of WEIGHT Variance
ANOVA of Squared Deviations from Group Means

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
GROUP	1	64108.8	64108.8	1.83	0.2246
Error	6	209888	34981.3		

Bartlett's Test for Homogeneity of WEIGHT Variance

Source	DF	Chi-Square	Pr > ChiSq
GROUP	1	2.4716	0.1159

Dunnett's t Tests for WEIGHT

NOTE: This test controls the Type I experimentwise error for comparisons of all treatments against a control.

Alpha	0.05
Error Degrees of Freedom	6
Error Mean Square	170.6894
Critical Value of Dunnett's t	2.44695

Comparisons significant at the 0.05 level are indicated by ***.

GROUP Comparison	Difference Between Means	Simultaneous 95% Confidence Limits	
REF - EXP	-6.346	-29.693	17.001

ANOVA FOR WEIGHT OF FROGS BETWEEN REFERENCE (REF) AND EXPERIMENTAL (EXP) SITES 433

-- Sex=M --

The ANOVA Procedure

Class Level Information

Class	Levels	Values
GROUP	2	EXP REF

Number of observations 8

Dependent Variable: WEIGHT

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	1	2.6671491	2.6671491	0.06	0.8143
Error	6	265.7568743	44.2928124		
Corrected Total	7	268.4240234			

R-Square	Coeff Var	Root MSE	WEIGHT Mean
0.009936	25.05318	6.655285	26.56463

Source	DF	Anova SS	Mean Square	F Value	Pr > F
GROUP	1	2.66714907	2.66714907	0.06	0.8143

Levene's Test for Homogeneity of WEIGHT Variance
ANOVA of Squared Deviations from Group Means

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
GROUP	1	5070.2	5070.2	4.36	0.0818
Error	6	6973.5	1162.3		

Bartlett's Test for Homogeneity of WEIGHT Variance

Source	DF	Chi-Square	Pr > ChiSq
GROUP	1	1.8380	0.1752

Dunnett's t Tests for WEIGHT

NOTE: This test controls the Type I experimentwise error for comparisons of all treatments against a control.

Alpha	0.05
Error Degrees of Freedom	6
Error Mean Square	44.29281
Critical Value of Dunnett's t	2.44695

Comparisons significant at the 0.05 level are indicated by ***.

GROUP Comparison	Difference Between Means	Simultaneous 95% Confidence Limits	
REF - EXP	-1.193	-13.086	10.700

-- Sex=F --

The NPAR1WAY Procedure

Wilcoxon Scores (Rank Sums) for Variable WEIGHT
Classified by Variable GROUP

GROUP	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
EXP	5	23.0	22.50	3.354102	4.600000
REF	3	13.0	13.50	3.354102	4.333333

Wilcoxon Two-Sample Test

Statistic	13.0000
Normal Approximation	
Z	0.0000
One-Sided Pr < Z	0.5000
Two-Sided Pr > |Z|	1.0000
t Approximation	
One-Sided Pr < Z	0.5000
Two-Sided Pr > |Z|	1.0000

Z includes a continuity correction of 0.5.

Kruskal-Wallis Test

Chi-Square	0.0222
DF	1
Pr > Chi-Square	0.8815

Median Scores (Number of Points Above Median) for Variable WEIGHT
Classified by Variable GROUP

GROUP	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
EXP	5	3.0	2.50	0.731925	0.600000
REF	3	1.0	1.50	0.731925	0.333333

Median Two-Sample Test

Statistic	1.0000
Z	-0.6831
One-Sided Pr < Z	0.2473
Two-Sided Pr > |Z|	0.4945

Median One-Way Analysis

Chi-Square	0.4667
DF	1
Pr > Chi-Square	0.4945

-- Sex=M --

The NPAR1WAY Procedure

Wilcoxon Scores (Rank Sums) for Variable WEIGHT
Classified by Variable GROUP

GROUP	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
EXP	5	25.0	22.50	3.354102	5.000000
REF	3	11.0	13.50	3.354102	3.666667

Wilcoxon Two-Sample Test

Statistic	11.0000
Normal Approximation	
Z	-0.5963
One-Sided Pr < Z	0.2755
Two-Sided Pr > |Z|	0.5510
t Approximation	
One-Sided Pr < Z	0.2849
Two-Sided Pr > |Z|	0.5698

Z includes a continuity correction of 0.5.

Kruskal-Wallis Test

Chi-Square	0.5556
DF	1
Pr > Chi-Square	0.4561

Median Scores (Number of Points Above Median) for Variable WEIGHT
Classified by Variable GROUP

GROUP	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
EXP	5	3.0	2.50	0.731925	0.600000
REF	3	1.0	1.50	0.731925	0.333333

Median Two-Sample Test

Statistic	1.0000
Z	-0.6831
One-Sided Pr < Z	0.2473
Two-Sided Pr > |Z|	0.4945

Median One-Way Analysis

Chi-Square	0.4667
DF	1
Pr > Chi-Square	0.4945

MEAN TESTICULAR WEIGHTS FOR FROGS COLLECTED BY POND 441

Obs	POND	GROUP	_TYPE_	_FREQ_	TESTES	T_SD	T_CV
1	C1	REF	0	20	0.024585	0.010550	42.9144
2	C3	REF	0	20	0.030953	0.023958	77.4001
3	C6	REF	0	20	0.047570	0.016667	35.0366
4	E1	EXP	0	10	0.036925	0.014242	38.5703
5	E3	EXP	0	10	0.053570	0.017665	32.9753
6	E4	EXP	0	10	0.057670	0.009618	16.6769
7	E6	EXP	0	10	0.033560	0.018074	53.8565
8	E8	EXP	0	10	0.044200	0.019885	44.9884

ANOVA FOR TESTICULAR WEIGHT FOR FROGS BETWEEN PONDS 442

The ANOVA Procedure

Class Level Information

Class	Levels	Values
POND	8	C1 C3 C6 E1 E3 E4 E6 E8

Number of observations 8

Dependent Variable: TESTES

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	7	0.00093152	0.00013307	.	.
Error	0	0.00000000	.		
Corrected Total	7	0.00093152			

R-Square	Coeff Var	Root MSE	TESTES Mean
1.000000	.	.	0.041129

Source	DF	Anova SS	Mean Square	F Value	Pr > F
POND	7	0.00093152	0.00013307	.	.

Levene's Test for Homogeneity of TESTES Variance
ANOVA of Squared Deviations from Group Means

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
POND	0	0	.	.	.
Error	0	0	.		

Bartlett's Test for Homogeneity of TESTES Variance

Source	DF	Chi-Square	Pr > ChiSq
POND	0	0	.

ANOVA FOR TESTICULAR WEIGHT FOR FROGS BETWEEN PONDS 445

The ANOVA Procedure

Level of POND	N	TESTES Mean	TESTES Std Dev
C1	1	0.02458500	.

C3	1	0.03095294	.
C6	1	0.04757000	.
E1	1	0.03692500	.
E3	1	0.05357000	.
E4	1	0.05767000	.
E6	1	0.03356000	.
E8	1	0.04420000	.

ANOVA FOR TESTICULAR WEIGHT FOR FROGS BETWEEN REFERENCE (REF) AND EXPERIMENTAL (EXP) SITE 446

The ANOVA Procedure

Class Level Information

Class	Levels	Values
GROUP	2	EXP REF

Number of observations 8

Dependent Variable: TESTES

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	1	0.00021934	0.00021934	1.85	0.2229
Error	6	0.00071218	0.00011870		
Corrected Total	7	0.00093152			

R-Square	Coeff Var	Root MSE	TESTES Mean
0.235460	26.48934	0.010895	0.041129

Source	DF	Anova SS	Mean Square	F Value	Pr > F
GROUP	1	0.00021934	0.00021934	1.85	0.2229

Levene's Test for Homogeneity of TESTES Variance
ANOVA of Squared Deviations from Group Means

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
GROUP	1	1.14E-10	1.14E-10	0.02	0.8818
Error	6	2.831E-8	4.719E-9		

Bartlett's Test for Homogeneity of TESTES Variance

Source	DF	Chi-Square	Pr > ChiSq
GROUP	1	0.0415	0.8386

Dunnett's t Tests for TESTES

NOTE: This test controls the Type I experimentwise error for comparisons of all treatments against a control.

Alpha	0.05
Error Degrees of Freedom	6
Error Mean Square	0.000119
Critical Value of Dunnett's t	2.44695

Comparisons significant at the 0.05 level are indicated by ***.

GROUP Comparison	Difference Between Means	Simultaneous 95% Confidence Limits	
REF - EXP	-0.010816	-0.030285	0.008653

The NPAR1WAY Procedure

Wilcoxon Scores (Rank Sums) for Variable TESTES
Classified by Variable GROUP

GROUP	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
EXP	5	27.0	22.50	3.354102	5.40
REF	3	9.0	13.50	3.354102	3.00

Wilcoxon Two-Sample Test

Statistic	9.0000
Normal Approximation	
Z	-1.1926
One-Sided Pr < Z	0.1165
Two-Sided Pr > |Z|	0.2330
t Approximation	
One-Sided Pr < Z	0.1359
Two-Sided Pr > |Z|	0.2719

Z includes a continuity correction of 0.5.

Kruskal-Wallis Test

Chi-Square	1.8000
DF	1
Pr > Chi-Square	0.1797

Median Scores (Number of Points Above Median) for Variable TESTES
Classified by Variable GROUP

GROUP	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
EXP	5	3.0	2.50	0.731925	0.600000
REF	3	1.0	1.50	0.731925	0.333333

Median Two-Sample Test

Statistic	1.0000
Z	-0.6831

One-Sided Pr < Z	0.2473
Two-Sided Pr > |Z|	0.4945

Median One-Way Analysis

Chi-Square	0.4667
DF	1
Pr > Chi-Square	0.4945

MEAN GONADOSOMATIC INDEX (GSI) OF FROGS COLLECTED BY POND AND SEX 452

Obs	POND	Sex	GROUP	_TYPE_	_FREQ_	GSI	GSI_SD	GSI_CV
1	C1	F	REF	0	20	1.20519	0.59529	49.3938
2	C1	M	REF	0	20	0.12568	0.02906	23.1225
3	C3	F	REF	0	20	2.32511	1.78135	76.6135
4	C3	M	REF	0	20	0.13554	0.04066	29.9998
5	C6	F	REF	0	20	1.56896	1.23447	78.6810
6	C6	M	REF	0	20	0.12992	0.03488	26.8432
7	E1	F	EXP	0	10	3.84820	3.58562	93.1766
8	E1	M	EXP	0	10	0.17529	0.04832	27.5681
9	E3	F	EXP	0	10	5.74161	2.38193	41.4853
10	E3	M	EXP	0	10	0.16518	0.03373	20.4210
11	E4	F	EXP	0	10	5.39806	1.45422	26.9397
12	E4	M	EXP	0	10	0.19433	0.03069	15.7908
13	E6	F	EXP	0	10	2.52463	1.94072	76.8714
14	E6	M	EXP	0	10	0.13001	0.04169	32.0636
15	E8	F	EXP	0	15	4.60581	3.38835	73.5669
16	E8	M	EXP	0	10	0.18583	0.02224	11.9674

ANOVA FOR GSI OF FROGS BETWEEN PONDS BY SEX 453

-- Sex=F --

The ANOVA Procedure

Class Level Information

Class	Levels	Values
POND	8	C1 C3 C6 E1 E3 E4 E6 E8

Number of observations 115

NOTE: Due to missing values, only 111 observations can be used in this analysis.

Dependent Variable: GSI

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	7	292.3774342	41.7682049	10.12	<.0001
Error	103	424.9868075	4.1260855		
Corrected Total	110	717.3642417			

R-Square	Coeff Var	Root MSE	GSI Mean
0.407572	68.15863	2.031277	2.980220

Source	DF	Anova SS	Mean Square	F Value	Pr > F
POND	7	292.3774342	41.7682049	10.12	<.0001

Levene's Test for Homogeneity of GSI Variance
ANOVA of Squared Deviations from Group Means

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
POND	7	1421.7	203.1	7.65	<.0001
Error	103	2732.9	26.5335		

Bartlett's Test for Homogeneity of GSI Variance

Source	DF	Chi-Square	Pr > ChiSq
POND	7	52.6276	<.0001

Dunnett's t Tests for GSI

NOTE: This test controls the Type I experimentwise error for comparisons of all treatments against a control.

Alpha	0.05
Error Degrees of Freedom	103
Error Mean Square	4.126086
Critical Value of Dunnett's t	2.69261

Comparisons significant at the 0.05 level are indicated by ***.

POND Comparison	Difference Between Means	Simultaneous 95% Confidence Limits		
E3 - C1	4.5364	2.4181	6.6547	***
E4 - C1	4.1929	2.0746	6.3112	***
E8 - C1	3.4006	1.5325	5.2688	***
E1 - C1	2.6430	0.0971	5.1889	***
E6 - C1	1.3194	-0.7989	3.4377	
C3 - C1	1.1199	-0.6097	2.8495	
C6 - C1	0.3638	-1.3658	2.0934	

ANOVA FOR GSI OF FROGS BETWEEN PONDS BY SEX 457

-- Sex=M --

The ANOVA Procedure

Class Level Information

Class	Levels	Values
POND	8	C1 C3 C6 E1 E3 E4 E6 E8

Number of observations 110

NOTE: Due to missing values, only 101 observations can be used in this analysis.

Dependent Variable: GSI

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	7	0.06119627	0.00874232	6.84	<.0001
Error	93	0.11878563	0.00127726		
Corrected Total	100	0.17998190			

R-Square	Coeff Var	Root MSE	GSI Mean
0.340013	24.34181	0.035739	0.146821

Source	DF	Anova SS	Mean Square	F Value	Pr > F
POND	7	0.06119627	0.00874232	6.84	<.0001

Levene's Test for Homogeneity of GSI Variance
ANOVA of Squared Deviations from Group Means

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
POND	7	0.000018	2.513E-6	0.86	0.5406
Error	93	0.000272	2.92E-6		

Bartlett's Test for Homogeneity of GSI Variance

Source	DF	Chi-Square	Pr > ChiSq
POND	7	5.9559	0.5449

Dunnett's t Tests for GSI

NOTE: This test controls the Type I experimentwise error for comparisons of all treatments against a control.

Alpha	0.05
Error Degrees of Freedom	93
Error Mean Square	0.001277
Critical Value of Dunnett's t	2.70384

Comparisons significant at the 0.05 level are indicated by ***.

POND Comparison	Difference Between Means	Simultaneous 95% Confidence Limits		
E4 - C1	0.06865	0.03122	0.10607	***
E8 - C1	0.06015	0.01517	0.10513	***
E1 - C1	0.04961	0.00918	0.09003	***
E3 - C1	0.03950	0.00208	0.07693	***
C3 - C1	0.00986	-0.02202	0.04173	
E6 - C1	0.00433	-0.03309	0.04176	
C6 - C1	0.00424	-0.02632	0.03480	

NONPARAMETRIC COMPARISON OF GSI BETWEEN PONDS BY SEX 461

-- Sex=F --

The NPAR1WAY Procedure

Wilcoxon Scores (Rank Sums) for Variable GSI
Classified by Variable POND

POND	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
C1	20	640.0	1120.0	130.332907	32.000000
C3	20	1005.0	1120.0	130.332907	50.250000
C6	20	780.0	1120.0	130.332907	39.000000
E1	6	347.0	336.0	76.681158	57.833333
E3	10	885.0	560.0	97.091023	88.500000
E4	10	887.0	560.0	97.091023	88.700000
E6	10	577.0	560.0	97.091023	57.700000
E8	15	1095.0	840.0	115.931014	73.000000

Kruskal-Wallis Test

Chi-Square	42.0856
DF	7
Pr > Chi-Square	<.0001

The NPAR1WAY Procedure

Median Scores (Number of Points Above Median) for Variable GSI
Classified by Variable POND

POND	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
C1	20	3.0	9.909910	2.033723	0.150000
C3	20	11.0	9.909910	2.033723	0.550000
C6	20	4.0	9.909910	2.033723	0.200000
E1	6	3.0	2.972973	1.196537	0.500000
E3	10	9.0	4.954955	1.515014	0.900000
E4	10	10.0	4.954955	1.515014	1.000000
E6	10	4.0	4.954955	1.515014	0.400000
E8	15	11.0	7.432432	1.808995	0.733333

Median One-Way Analysis

Chi-Square	36.9250
DF	7

```
Pr > Chi-Square     <.0001
```

-- Sex=M --

The NPAR1WAY Procedure

Wilcoxon Scores (Rank Sums) for Variable GSI
Classified by Variable POND

POND	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
C1	20	714.0	1020.0	117.345643	35.700000
C3	17	783.0	867.0	110.172592	46.058824
C6	20	757.0	1020.0	117.345643	37.850000
E1	8	548.0	408.0	79.523581	68.500000
E3	10	645.0	510.0	87.948849	64.500000
E4	10	829.0	510.0	87.948849	82.900000
E6	10	394.0	510.0	87.948849	39.400000
E8	6	481.0	306.0	69.606034	80.166667

Kruskal-Wallis Test

Chi-Square	34.3083
DF	7
Pr > Chi-Square	<.0001

Median Scores (Number of Points Above Median) for Variable GSI
Classified by Variable POND

POND	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
C1	20	5.0	9.900990	2.012363	0.250000
C3	17	9.0	8.415842	1.889352	0.529412
C6	20	4.0	9.900990	2.012363	0.200000
E1	8	6.0	3.960396	1.363751	0.750000
E3	10	6.0	4.950495	1.508236	0.600000
E4	10	10.0	4.950495	1.508236	1.000000
E6	10	4.0	4.950495	1.508236	0.400000
E8	6	6.0	2.970297	1.193675	1.000000

Median One-Way Analysis

Chi-Square	30.7445
DF	7
Pr > Chi-Square	<.0001

ANOVA FOR GSI OF FROGS BETWEEN REFERENCE (REF) AND EXPERIMENTAL (EXP) SITES 465

-- Sex=F --

The ANOVA Procedure

Class Level Information

Class	Levels	Values
GROUP	2	EXP REF

Number of observations 8

Dependent Variable: GSI

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	1	13.91190688	13.91190688	11.42	0.0149
Error	6	7.30985217	1.21830869		
Corrected Total	7	21.22175905			

R-Square	Coeff Var	Root MSE	GSI Mean
0.655549	32.44289	1.103770	3.402195

Source	DF	Anova SS	Mean Square	F Value	Pr > F
GROUP	1	13.91190688	13.91190688	11.42	0.0149

Levene's Test for Homogeneity of GSI Variance
ANOVA of Squared Deviations from Group Means

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
GROUP	1	2.3261	2.3261	1.69	0.2409
Error	6	8.2425	1.3737		

Bartlett's Test for Homogeneity of GSI Variance

Source	DF	Chi-Square	Pr > ChiSq
GROUP	1	1.1609	0.2813

Dunnett's t Tests for GSI

NOTE: This test controls the Type I experimentwise error for comparisons of all treatments against a control.

Alpha	0.05
Error Degrees of Freedom	6
Error Mean Square	1.218309
Critical Value of Dunnett's t	2.44695

Comparisons significant at the 0.05 level are indicated by ***.

GROUP Comparison	Difference Between Means	Simultaneous 95% Confidence Limits		
REF - EXP	-2.7239	-4.6963	-0.7515	***

ANOVA FOR GSI OF FROGS BETWEEN REFERENCE (REF) AND EXPERIMENTAL (EXP) SITES 469

-- Sex=M --

The ANOVA Procedure

Class Level Information

Class	Levels	Values
GROUP	2	EXP REF

Number of observations 8

Dependent Variable: GSI

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	1	0.00296251	0.00296251	6.99	0.0383
Error	6	0.00254148	0.00042358		
Corrected Total	7	0.00550399			

R-Square	Coeff Var	Root MSE	GSI Mean
0.538248	13.25886	0.020581	0.155225

Source	DF	Anova SS	Mean Square	F Value	Pr > F
GROUP	1	0.00296251	0.00296251	6.99	0.0383

Levene's Test for Homogeneity of GSI Variance
ANOVA of Squared Deviations from Group Means

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
GROUP	1	4.36E-7	4.36E-7	1.49	0.2676
Error	6	1.753E-6	2.921E-7		

Bartlett's Test for Homogeneity of GSI Variance

Source	DF	Chi-Square	Pr > ChiSq
GROUP	1	3.4829	0.0620

Dunnett's t Tests for GSI

NOTE: This test controls the Type I experimentwise error for comparisons of all treatments against a control.

Alpha	0.05
Error Degrees of Freedom	6
Error Mean Square	0.000424
Critical Value of Dunnett's t	2.44695

Comparisons significant at the 0.05 level are indicated by ***.

GROUP Comparison	Difference Between Means	Simultaneous 95% Confidence Limits		
REF - EXP	-0.03975	-0.07653	-0.00297	***

--- Sex=F ---

The NPAR1WAY Procedure

Wilcoxon Scores (Rank Sums) for Variable GSI
Classified by Variable GROUP

GROUP	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
EXP	5	30.0	22.50	3.354102	6.0
REF	3	6.0	13.50	3.354102	2.0

Wilcoxon Two-Sample Test

Statistic	6.0000
Normal Approximation	
Z	-2.0870
One-Sided Pr < Z	0.0184
Two-Sided Pr > |Z|	0.0369
t Approximation	
One-Sided Pr < Z	0.0377
Two-Sided Pr > |Z|	0.0753

Z includes a continuity correction of 0.5.

Kruskal-Wallis Test

Chi-Square	5.0000
DF	1
Pr > Chi-Square	0.0253

Median Scores (Number of Points Above Median) for Variable GSI
Classified by Variable GROUP

GROUP	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
EXP	5	4.0	2.50	0.731925	0.80
REF	3	0.0	1.50	0.731925	0.00

Median Two-Sample Test

Statistic	0.0000
Z	-2.0494
One-Sided Pr < Z	0.0202
Two-Sided Pr > |Z|	0.0404

Median One-Way Analysis

Chi-Square	4.2000
DF	1
Pr > Chi-Square	0.0404

--- Sex=M ---

The NPAR1WAY Procedure

Wilcoxon Scores (Rank Sums) for Variable GSI
Classified by Variable GROUP

GROUP	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
EXP	5	29.0	22.50	3.354102	5.800000
REF	3	7.0	13.50	3.354102	2.333333

Wilcoxon Two-Sample Test

Statistic	7.0000
Normal Approximation	
Z	-1.7889
One-Sided Pr < Z	0.0368
Two-Sided Pr > |Z|	0.0736
t Approximation	
One-Sided Pr < Z	0.0584
Two-Sided Pr > |Z|	0.1168

Z includes a continuity correction of 0.5.

Kruskal-Wallis Test

Chi-Square	3.7556
DF	1
Pr > Chi-Square	0.0526

The NPAR1WAY Procedure

Median Scores (Number of Points Above Median) for Variable GSI
Classified by Variable GROUP

GROUP	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
EXP	5	4.0	2.50	0.731925	0.80
REF	3	0.0	1.50	0.731925	0.00

Median Two-Sample Test

Statistic	0.0000
Z	-2.0494
One-Sided Pr < Z	0.0202
Two-Sided Pr > |Z|	0.0404

Median One-Way Analysis

Chi-Square	4.2000
DF	1
Pr > Chi-Square	0.0404

MEAN CONDITION INDEX OF FROGS COLLECTED BY POND AND SEX 477

Obs	POND	Sex	GROUP	_TYPE_	_FREQ_	CONDITION	CI_SD	CI_CV
1	C1	F	REF	0	20	0.46055	0.10789	23.4271
2	C1	M	REF	0	20	0.32900	0.06735	20.4721
3	C3	F	REF	0	20	0.47447	0.19746	41.6174
4	C3	M	REF	0	20	0.33864	0.14453	42.6786
5	C6	F	REF	0	20	0.53024	0.09217	17.3826
6	C6	M	REF	0	20	0.50706	0.10983	21.6600
7	E1	F	EXP	0	10	0.41857	0.18672	44.6087
8	E1	M	EXP	0	10	0.37327	0.15799	42.3275
9	E3	F	EXP	0	10	0.52443	0.06631	12.6435
10	E3	M	EXP	0	10	0.48108	0.09040	18.7901
11	E4	F	EXP	0	10	0.61236	0.14012	22.8822
12	E4	M	EXP	0	10	0.46696	0.04620	9.8932
13	E6	F	EXP	0	10	0.74118	0.17227	23.2423
14	E6	M	EXP	0	10	0.40045	0.12809	31.9870
15	E8	F	EXP	0	15	0.42604	0.19041	44.6937
16	E8	M	EXP	0	10	0.37014	0.11131	30.0735

ANOVA FOR CONDITION INDEX OF FROGS BETWEEN PONDS BY SEX 478

--- Sex=F ---

The ANOVA Procedure

Class Level Information

Class	Levels	Values
POND	8	C1 C3 C6 E1 E3 E4 E6 E8

Number of observations 115

NOTE: Due to missing values, only 111 observations can be used in this analysis.

Dependent Variable: CI

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	7	0.87825562	0.12546509	5.65	<.0001
Error	103	2.28870294	0.02222042		
Corrected Total	110	3.16695856			

R-Square	Coeff Var	Root MSE	CI Mean
0.277318	29.03514	0.149065	0.513396

Source	DF	Anova SS	Mean Square	F Value	Pr > F
POND	7	0.87825562	0.12546509	5.65	<.0001

Levene's Test for Homogeneity of CI Variance
ANOVA of Squared Deviations from Group Means

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
POND	7	0.0167	0.00238	3.16	0.0046
Error	103	0.0775	0.000753		

Bartlett's Test for Homogeneity of CI Variance

Source	DF	Chi-Square	Pr > ChiSq
POND	7	23.0265	0.0017

Dunnett's t Tests for CI

NOTE: This test controls the Type I experimentwise error for comparisons of all treatments against a control.

Alpha	0.05
Error Degrees of Freedom	103
Error Mean Square	0.02222
Critical Value of Dunnett's t	2.69261

Comparisons significant at the 0.05 level are indicated by ***.

POND Comparison	Difference Between Means	Simultaneous 95% Confidence Limits		
E6 - C1	0.28063	0.12518	0.43608	***
E4 - C1	0.15181	-0.00365	0.30726	
C6 - C1	0.06969	-0.05723	0.19662	
E3 - C1	0.06388	-0.09157	0.21933	
C3 - C1	0.01392	-0.11300	0.14085	
E8 - C1	-0.03451	-0.17161	0.10258	
E1 - C1	-0.04198	-0.22881	0.14485	

ANOVA FOR CONDITION INDEX OF FROGS BETWEEN PONDS BY SEX 482

---------- Sex=M ----------

The ANOVA Procedure

Class Level Information

Class	Levels	Values
POND	8	C1 C3 C6 E1 E3 E4 E6 E8

Number of observations 110

NOTE: Due to missing values, only 101 observations can be used in this analysis.

Dependent Variable: CI

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	7	0.50980220	0.07282889	6.01	<.0001
Error	93	1.12670273	0.01211508		
Corrected Total	100	1.63650493			

R-Square	Coeff Var	Root MSE	CI Mean
0.311519	27.00248	0.110069	0.407624

Source	DF	Anova SS	Mean Square	F Value	Pr > F
POND	7	0.50980220	0.07282889	6.01	<.0001

Levene's Test for Homogeneity of CI Variance
ANOVA of Squared Deviations from Group Means

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
POND	7	0.00421	0.000602	1.65	0.1310
Error	93	0.0339	0.000365		

Bartlett's Test for Homogeneity of CI Variance

Source	DF	Chi-Square	Pr > ChiSq
POND	7	20.4868	0.0046

Dunnett's t Tests for CI

NOTE: This test controls the Type I experimentwise error for comparisons of all treatments against a control.

Alpha	0.05
Error Degrees of Freedom	93
Error Mean Square	0.012115
Critical Value of Dunnett's t	2.70384

Comparisons significant at the 0.05 level are indicated by ***.

POND Comparison	Difference Between Means	Simultaneous 95% Confidence Limits		
C6 - C1	0.17805	0.08394	0.27217	***
E3 - C1	0.15208	0.03682	0.26734	***
E4 - C1	0.13795	0.02269	0.25322	***
E6 - C1	0.07145	-0.04381	0.18672	
E1 - C1	0.04426	-0.08024	0.16876	
E8 - C1	0.04114	-0.09739	0.17967	
C3 - C1	0.00964	-0.08853	0.10782	

--- Sex=F ---

The NPAR1WAY Procedure

Wilcoxon Scores (Rank Sums) for Variable CI
Classified by Variable POND

POND	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
C1	20	901.0	1120.0	130.332907	45.050000
C3	20	982.0	1120.0	130.332907	49.100000
C6	20	1200.0	1120.0	130.332907	60.000000
E1	6	216.0	336.0	76.681158	36.000000
E3	10	607.0	560.0	97.091023	60.700000
E4	10	762.0	560.0	97.091023	76.200000
E6	10	946.0	560.0	97.091023	94.600000
E8	15	602.0	840.0	115.931014	40.133333

Kruskal-Wallis Test

Chi-Square	28.0380
DF	7
Pr > Chi-Square	0.0002

Median Scores (Number of Points Above Median) for Variable CI
Classified by Variable POND

POND	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
C1	20	7.0	9.909910	2.033723	0.350000
C3	20	8.0	9.909910	2.033723	0.400000
C6	20	11.0	9.909910	2.033723	0.550000
E1	6	1.0	2.972973	1.196537	0.166667
E3	10	6.0	4.954955	1.515014	0.600000
E4	10	8.0	4.954955	1.515014	0.800000
E6	10	10.0	4.954955	1.515014	1.000000
E8	15	4.0	7.432432	1.808995	0.266667

Median One-Way Analysis

Chi-Square	22.5214
DF	7
Pr > Chi-Square	0.0021

NONPARAMETRIC COMPARISON OF CONDITION INDEX BETWEEN PONDS BY SEX 488

-- Sex=M --

The NPAR1WAY Procedure

Wilcoxon Scores (Rank Sums) for Variable CI
Classified by Variable POND

POND	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
C1	20	632.0	1020.0	117.345643	31.600000
C3	17	572.0	867.0	110.172592	33.647059
C6	20	1476.0	1020.0	117.345643	73.800000
E1	8	319.0	408.0	79.523581	39.875000
E3	10	701.0	510.0	87.948849	70.100000
E4	10	700.0	510.0	87.948849	70.000000
E6	10	480.0	510.0	87.948849	48.000000
E8	6	271.0	306.0	69.606034	45.166667

Kruskal-Wallis Test

Chi-Square	36.7915
DF	7
Pr > Chi-Square	<.0001

The NPAR1WAY Procedure

Median Scores (Number of Points Above Median) for Variable CI
Classified by Variable POND

POND	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
C1	20	2.0	9.900990	2.012363	0.100000
C3	17	4.0	8.415842	1.889352	0.235294
C6	20	17.0	9.900990	2.012363	0.850000
E1	8	3.0	3.960396	1.363751	0.375000
E3	10	7.0	4.950495	1.508236	0.700000
E4	10	10.0	4.950495	1.508236	1.000000
E6	10	3.0	4.950495	1.508236	0.300000
E8	6	4.0	2.970297	1.193675	0.666667

Median One-Way Analysis

Chi-Square	41.3124
DF	7

```
Pr > Chi-Square      <.0001
```

ANOVA FOR CONDITION INDEX OF FROGS BETWEEN REFERENCE (REF) AND EXPERIMENTAL (EXP) SITES 490

--- Sex=F ---

The ANOVA Procedure

Class Level Information

Class	Levels	Values
GROUP	2	EXP REF

Number of observations 8

Dependent Variable: CONDITION

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	1	0.00589954	0.00589954	0.46	0.5212
Error	6	0.07630211	0.01271702		
Corrected Total	7	0.08220165			

R-Square	Coeff Var	Root MSE	CONDITION Mean
0.071769	21.54234	0.112770	0.523480

Source	DF	Anova SS	Mean Square	F Value	Pr > F
GROUP	1	0.00589954	0.00589954	0.46	0.5212

Levene's Test for Homogeneity of CONDITION Variance
ANOVA of Squared Deviations from Group Means

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
GROUP	1	0.000358	0.000358	2.43	0.1703
Error	6	0.000884	0.000147		

Bartlett's Test for Homogeneity of CONDITION Variance

Source	DF	Chi-Square	Pr > ChiSq
GROUP	1	2.5067	0.1134

Dunnett's t Tests for CONDITION

NOTE: This test controls the Type I experimentwise error for comparisons of all treatments against a control.

Alpha	0.05
Error Degrees of Freedom	6
Error Mean Square	0.012717
Critical Value of Dunnett's t	2.44695

Comparisons significant at the 0.05 level are indicated by ***.

GROUP Comparison	Difference Between Means	Simultaneous 95% Confidence Limits	
REF - EXP	-0.05609	-0.25761	0.14543

ANOVA FOR CONDITION INDEX OF FROGS BETWEEN REFERENCE (REF) AND EXPERIMENTAL (EXP) SITES 494

-- Sex=M --

The ANOVA Procedure

Class Level Information

Class	Levels	Values
GROUP	2	EXP REF

Number of observations 8

Dependent Variable: CONDITION

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	1	0.00134800	0.00134800	0.26	0.6279
Error	6	0.03102745	0.00517124		
Corrected Total	7	0.03237545			

R-Square	Coeff Var	Root MSE	CONDITION Mean
0.041636	17.61129	0.071911	0.408325

Source	DF	Anova SS	Mean Square	F Value	Pr > F
GROUP	1	0.00134800	0.00134800	0.26	0.6279

Levene's Test for Homogeneity of CONDITION Variance
ANOVA of Squared Deviations from Group Means

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
GROUP	1	0.000038	0.000038	3.08	0.1298
Error	6	0.000074	0.000012		

Bartlett's Test for Homogeneity of CONDITION Variance

Source	DF	Chi-Square	Pr > ChiSq
GROUP	1	1.0139	0.3140

Dunnett's t Tests for CONDITION

NOTE: This test controls the Type I experimentwise error for comparisons of all treatments against a control.

Alpha	0.05
Error Degrees of Freedom	6
Error Mean Square	0.005171
Critical Value of Dunnett's t	2.44695

Comparisons significant at the 0.05 level are indicated by ***.

GROUP Comparison	Difference Between Means	Simultaneous 95% Confidence Limits	
REF - EXP	-0.02681	-0.15532	0.10169

-- Sex=F --

The NPAR1WAY Procedure

Wilcoxon Scores (Rank Sums) for Variable CONDITION
Classified by Variable GROUP

GROUP	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
EXP	5	23.0	22.50	3.354102	4.600000
REF	3	13.0	13.50	3.354102	4.333333

Wilcoxon Two-Sample Test

Statistic	13.0000
Normal Approximation	
Z	0.0000
One-Sided Pr < Z	0.5000
Two-Sided Pr > |Z|	1.0000
t Approximation	
One-Sided Pr < Z	0.5000
Two-Sided Pr > |Z|	1.0000

Z includes a continuity correction of 0.5.

Kruskal-Wallis Test

Chi-Square	0.0222
DF	1
Pr > Chi-Square	0.8815

The NPAR1WAY Procedure

Median Scores (Number of Points Above Median) for Variable CONDITION
Classified by Variable GROUP

GROUP	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
EXP	5	3.0	2.50	0.731925	0.600000
REF	3	1.0	1.50	0.731925	0.333333

Median Two-Sample Test

Statistic	1.0000
Z	-0.6831
One-Sided Pr < Z	0.2473
Two-Sided Pr > |Z|	0.4945

Median One-Way Analysis

Chi-Square	0.4667
DF	1
Pr > Chi-Square	0.4945

-- Sex=M --

The NPAR1WAY Procedure

Wilcoxon Scores (Rank Sums) for Variable CONDITION
Classified by Variable GROUP

GROUP	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
EXP	5	25.0	22.50	3.354102	5.000000
REF	3	11.0	13.50	3.354102	3.666667

Wilcoxon Two-Sample Test

Statistic	11.0000
Normal Approximation	
Z	-0.5963
One-Sided Pr < Z	0.2755
Two-Sided Pr > |Z|	0.5510
t Approximation	
One-Sided Pr < Z	0.2849
Two-Sided Pr > |Z|	0.5698

Z includes a continuity correction of 0.5.

Kruskal-Wallis Test

Chi-Square	0.5556
DF	1
Pr > Chi-Square	0.4561

The NPAR1WAY Procedure

Median Scores (Number of Points Above Median) for Variable CONDITION
Classified by Variable GROUP

GROUP	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
EXP	5	3.0	2.50	0.731925	0.600000
REF	3	1.0	1.50	0.731925	0.333333

Median Two-Sample Test

Statistic	1.0000
Z	-0.6831
One-Sided Pr < Z	0.2473
Two-Sided Pr > |Z|	0.4945

Median One-Way Analysis

Chi-Square	0.4667
DF	1
Pr > Chi-Square	0.4945

MEAN FEMALE PLASMA ESTRADIOL CONCENTRATIONS BY SITE AND ANIMAL NUMBER

Obs	Site	Ind_No	_TYPE_	_FREQ_	E2	STD	CV
1	C1	21	0	3	4748.49	330.25	6.9549
2	C1	22	0	3	97.48	21.83	22.3947
3	C1	23	0	3	22.77	12.41	54.5215
4	C1	24	0	3	33025.69	7581.68	22.9569
5	C1	25	0	3	51.59	27.63	53.5657
6	C1	26	0	3	3137.18	267.56	8.5286
7	C1	27	0	3	3877.23	780.16	20.1215
8	C1	28	0	3	13.78	2.62	19.0307
9	C1	29	0	3	2781.17	285.40	10.2618
10	C1	30	0	3	110.61	25.45	23.0079
11	C1	31	0	3	25.21	10.25	40.6502
12	C1	32	0	3	5463.82	1002.76	18.3527
13	C1	33	0	3	49.35	24.61	49.8719
14	C1	34	0	3	292.85	184.51	63.0053
15	C1	35	0	3	55.43	25.69	46.3413
16	C1	36	0	3	6696.34	1406.15	20.9987
17	C1	37	0	3	.	.	.
18	C1	38	0	3	29043.30	0.00	0.0000
19	C1	39	0	3	16585.36	2876.22	17.3419
20	C1	40	0	3	14608.67	1333.87	9.1307
21	C3	21	0	3	18963.76	6968.94	36.7487
22	C3	22	0	3	69.64	8.83	12.6770
23	C3	23	0	3	242.59	62.08	25.5892
24	C3	24	0	3	7865.85	343.34	4.3649
25	C3	25	0	3	.	.	.
26	C3	26	0	3	367.45	87.33	23.7661
27	C3	27	0	3	6028.80	244.98	4.0635
28	C3	28	0	3	.	.	.
29	C3	29	0	3	145.50	58.21	40.0054
30	C3	30	0	3	6402.32	782.25	12.2182
31	C3	31	0	3	8629.88	1789.24	20.7330
32	C3	32	0	3	5017.56	551.41	10.9895
33	C3	33	0	3	33218.85	0.00	0.0000
34	C3	34	0	3	33.97	3.34	9.8194
35	C3	35	0	3	23.23	10.79	46.4362
36	C3	36	0	3	249.98	8.50	3.4014
37	C3	37	0	3	169.56	36.07	21.2708
38	C3	38	0	3	15342.62	4160.93	27.1200
39	C3	39	0	3	5834.65	1516.16	25.9854
40	C3	40	0	3	108.12	63.16	58.4186
41	C6	21	0	3	8718.85	770.64	8.8388
42	C6	22	0	3	112.90	30.66	27.1571
43	C6	23	0	3	.	.	.
44	C6	24	0	3	4578.96	512.44	11.1912
45	C6	25	0	3	546.55	199.05	36.4200
46	C6	26	0	3	26.31	19.35	73.5299

47	C6	27	0	3	4376.85	163.55	3.7366
48	C6	28	0	3	31.89	5.44	17.0485
49	C6	29	0	3	5682.53	431.56	7.5946
50	C6	30	0	3	4741.45	502.20	10.5918
51	C6	31	0	3	73.75	21.62	29.3201
52	C6	32	0	3	3646.29	166.95	4.5785
53	C6	33	0	3	4181.47	.	.
54	C6	34	0	3	19.63	16.47	83.9291
55	C6	35	0	3	26.74	14.71	55.0063
56	C6	36	0	3	56754.90	0.00	0.0000
57	C6	37	0	3	4586.95	357.81	7.8006
58	C6	38	0	3	34923.37	1076.57	3.0827
59	C6	39	0	3	7233.54	943.02	13.0367
60	C6	40	0	3	105573.43	9858.48	9.3380
61	E1	1	0	3	260.32	44.45	17.0755
62	E1	2	0	3	1859.49	92.60	4.9797
63	E1	3	0	3	3229.19	374.05	11.5835
64	E1	4	0	3	680.29	79.79	11.7284
65	E1	5	0	3	46.21	19.26	41.6735
66	E1	6	0	3	.	.	.
67	E3	1	0	3	128.90	23.59	18.3008
68	E3	2	0	3	198.40	45.41	22.8901
69	E3	3	0	3	162.61	19.74	12.1382
70	E3	4	0	3	266.59	4.57	1.7142
71	E3	5	0	3	.	.	.

MEAN FEMALE PLASMA ESTRADIOL CONCENTRATIONS BY SITE AND ANIMAL NUMBER

Obs	Site	Ind_No	_TYPE_	_FREQ_	E2	STD	CV
72	E3	6	0	3	206.28	25.87	12.5389
73	E3	7	0	3	89.89	11.12	12.3714
74	E3	8	0	3	296.97	13.75	4.6306
75	E3	9	0	3	367.43	44.06	11.9904
76	E3	10	0	3	122.72	13.99	11.3976
77	E4	1	0	3	1041.18	134.13	12.8821
78	E4	2	0	3	2967.96	495.96	16.7104
79	E4	3	0	3	1810.90	319.77	17.6580
80	E4	4	0	3	659.32	146.41	22.2065
81	E4	5	0	3	561.15	83.25	14.8360
82	E4	6	0	3	3533.16	209.97	5.9427
83	E4	7	0	3	781.20	25.54	3.2692
84	E4	8	0	3	76.74	4.14	5.3991
85	E4	9	0	3	7830.98	1283.47	16.3897
86	E4	10	0	3	2956.11	376.50	12.7363
87	E6	1	0	3	95.92	40.27	41.9873
88	E6	2	0	3	124.57	27.11	21.7657
89	E6	3	0	3	7.63	0.00	0.0000
90	E6	4	0	3	138.21	47.09	34.0689
91	E6	5	0	3	87.17	2.84	3.2539
92	E6	6	0	3	166.49	32.02	19.2329
93	E6	7	0	3	182.97	58.74	32.1016
94	E6	8	0	3	10.56	0.00	0.0000
95	E6	9	0	3	140.54	29.13	20.7254
96	E6	10	0	3	128.53	31.29	24.3410
97	E8	1	0	3	392.23	31.03	7.9118
98	E8	2	0	3	1894.28	290.91	15.3573
99	E8	3	0	3	248.29	16.21	6.5302
100	E8	4	0	3	739.52	140.60	19.0130
101	E8	5	0	3	3581.29	223.32	6.2359
102	E8	6	0	3	594.92	53.15	8.9345
103	E8	7	0	3	567.99	61.997	10.9152
104	E8	8	0	3	8292.57	562.256	6.7802
105	E8	9	0	3	253.48	13.662	5.3897
106	E8	10	0	3	245.23	35.042	14.2896
107	E8	11	0	3	490.67	159.633	32.5339
108	E8	12	0	3	114.38	23.707	20.7265
109	E8	13	0	3	.	.	.
110	E8	14	0	3	.	.	.
111	E8	15	0	3	.	.	.

ANOVA FOR FEMALE PLASMA ESTRADIOL BETWEEN SITES 85

The ANOVA Procedure

Class Level Information

Class	Levels	Values
Site	8	C1 C3 C6 E1 E3 E4 E6 E8

Number of observations 111

NOTE: Due to missing values, only 102 observations can be used in this analysis.

Dependent Variable: E2

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	7	1995951818	285135974	1.69	0.1218
Error	94	15898863008	169136841		
Corrected Total	101	17894814826			

R-Square	Coeff Var	Root MSE	E2 Mean
0.111538	253.2194	13005.26	5135.965

Source	DF	Anova SS	Mean Square	F Value	Pr > F
Site	7	1995951818	285135974	1.69	0.1218

Levene's Test for Homogeneity of E2 Variance
ANOVA of Squared Deviations from Group Means

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Site	7	6.25E18	8.929E17	1.19	0.3154
Error	94	7.045E19	7.495E17		

Bartlett's Test for Homogeneity of E2 Variance

Source	DF	Chi-Square	Pr > ChiSq
Site	7	250.4	<.0001

Dunnett's t Tests for E2

NOTE: This test controls the Type I experimentwise error for comparisons of all treatments against a control.

Alpha	0.05
Error Degrees of Freedom	94
Error Mean Square	1.6914E8
Critical Value of Dunnett's t	2.69946

Comparisons significant at the 0.05 level are indicated by ***.

Site Comparison	Difference Between Means	Simultaneous 95% Confidence Limits	
C6 - C1	6587	-4803	17977
C3 - C1	-312	-11860	11235
E4 - C1	-4130	-17846	9586
E8 - C1	-4901	-17846	8045
E1 - C1	-5137	-22783	12509
E3 - C1	-6147	-20354	8059
E6 - C1	-6244	-19959	7472

The NPAR1WAY Procedure

Wilcoxon Scores (Rank Sums) for Variable E2
Classified by Variable Site

Site	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
C1	19	985.0	978.50	116.343958	51.842105
C3	18	1059.0	927.00	113.921025	58.833333
C6	19	1142.0	978.50	116.343958	60.105263
E1	5	247.0	257.50	64.520669	49.400000
E3	9	331.0	463.50	84.759955	36.777778
E4	10	603.0	515.00	88.863191	60.300000
E6	10	235.0	515.00	88.863191	23.500000
E8	12	651.0	618.00	96.280839	54.250000

Kruskal-Wallis Test

Chi-Square	14.9116
DF	7
Pr > Chi-Square	0.0371

Median Scores (Number of Points Above Median) for Variable E2
Classified by Variable Site

Site	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
C1	19	10.0	9.50	1.975719	0.526316
C3	18	9.0	9.00	1.934573	0.500000
C6	19	13.0	9.50	1.975719	0.684211
E1	5	3.0	2.50	1.095671	0.600000
E3	9	0.0	4.50	1.439369	0.000000
E4	10	9.0	5.00	1.509049	0.900000
E6	10	0.0	5.00	1.509049	0.000000
E8	12	7.0	6.00	1.635013	0.583333

Median One-Way Analysis

Chi-Square	28.2849
DF	7
Pr > Chi-Square	0.0002

FEMALE PLASMA ESTRADIOL CONCENTRATIONS BY TREATMENT 91

Obs	TREAT	_TYPE_	_FREQ_	E2	STD	CV
1	ATRAZINE	0	153	1057.20	1810.05	171.211
2	CONTROL	0	180	8612.35	17414.02	202.198

ANOVA FOR FEMALE PLASMA ESTRADIOL ACROSS TREATMENTS 92

The ANOVA Procedure
Class Level Information

Class	Levels	Values
TREAT	2	ATRAZINE CONTROL

Number of observations 333

NOTE: Due to missing values, only 302 observations can be used in this analysis.

Dependent Variable: E2

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	1	4277610072	4277610072	25.73	<.0001
Error	300	49878301383	166261005		
Corrected Total	301	54155911454			

R-Square	Coeff Var	Root MSE	E2 Mean
0.078987	249.8882	12894.22	5159.997

Source	DF	Anova SS	Mean Square	F Value	Pr > F
TREAT	1	4277610072	4277610072	25.73	<.0001

Levene's Test for Homogeneity of E2 Variance
ANOVA of Squared Deviations from Group Means

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
TREAT	1	6.662E18	6.662E18	7.08	0.0082
Error	300	2.821E20	9.404E17		

Bartlett's Test for Homogeneity of E2 Variance

Source	DF	Chi-Square	Pr > ChiSq
TREAT	1	438.5	<.0001

Dunnett's t Tests for E2

NOTE: This test controls the Type I experimentwise error for comparisons of all treatments against a control.

Alpha	0.05
Error Degrees of Freedom	300
Error Mean Square	1.6626E8
Critical Value of Dunnett's t	1.96790

Comparisons significant at the 0.05 level are indicated by ***.

TREAT Comparison	Difference Between Means	Simultaneous 95% Confidence Limits		
CONTROL - ATRAZINE	7555	4624	10486	***

NONPARAMETRIC COMPARISON OF FEMALE PLASMA ESTRADIOL BETWEEN TREATMENTS 96

The NPAR1WAY Procedure

Wilcoxon Scores (Rank Sums) for Variable E2
Classified by Variable TREAT

TREAT	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
ATRAZINE	138	18405.0	20907.0	755.946352	133.369565
CONTROL	164	27348.0	24846.0	755.946352	166.756098

Average scores were used for ties.

Wilcoxon Two-Sample Test

Statistic	18405.0000
Normal Approximation	
Z	-3.3091
One-Sided Pr < Z	0.0005
Two-Sided Pr > |Z|	0.0009
t Approximation	
One-Sided Pr < Z	0.0005
Two-Sided Pr > |Z|	0.0010

Z includes a continuity correction of 0.5.

Kruskal-Wallis Test

Chi-Square	10.9545
DF	1
Pr > Chi-Square	0.0009

Median Scores (Number of Points Above Median) for Variable E2
Classified by Variable TREAT

TREAT	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
ATRAZINE	138	57.0	69.0	4.335590	0.413043
CONTROL	164	94.0	82.0	4.335590	0.573171

Average scores were used for ties.

Median Two-Sample Test

Statistic	57.0000
Z	-2.7678
One-Sided Pr < Z	0.0028
Two-Sided Pr > |Z|	0.0056

Median One-Way Analysis

Chi-Square	7.6607
DF	1
Pr > Chi-Square	0.0056

MEAN FEMALE PLASMA TESTOSTERONE CONCENTRATIONS BY SITE AND ANIMAL NUMBER

Obs	Site	Ind_No	TREAT	_TYPE_	_FREQ_	T	STD	CV
1	C1	21	CONTROL	0	6	1934.52	262.42	13.565
2	C1	22	CONTROL	0	6	321.86	293.90	91.313
3	C1	23	CONTROL	0	6	86.37	34.90	40.408
4	C1	24	CONTROL	0	6	14971.64	3255.59	21.745
5	C1	25	CONTROL	0	6	139.76	121.89	87.217
6	C1	26	CONTROL	0	6	103.94	85.34	82.100
7	C1	27	CONTROL	0	6	7023.04	900.09	12.816
8	C1	28	CONTROL	0	6	120.39	143.49	119.182
9	C1	29	CONTROL	0	6	3169.52	186.57	5.887
10	C1	30	CONTROL	0	6	173.79	21.49	12.363
11	C1	31	CONTROL	0	6	21.74	4.23	19.473
12	C1	32	CONTROL	0	6	2863.16	374.89	13.094
13	C1	33	CONTROL	0	6	7710.56	11725.47	152.070
14	C1	34	CONTROL	0	6	882.00	742.98	84.238
15	C1	35	CONTROL	0	6	19.60	6.26	31.937
16	C1	36	CONTROL	0	6	2153.11	262.17	12.176
17	C1	37	CONTROL	0	6	.	.	.
18	C1	38	CONTROL	0	6	14521.65	0.00	0.000
19	C1	39	CONTROL	0	6	6802.20	479.18	7.045
20	C1	40	CONTROL	0	6	4473.92	192.11	4.294
21	C3	21	CONTROL	0	6	7195.61	2147.40	29.843
22	C3	22	CONTROL	0	6	182.50	51.98	28.479
23	C3	23	CONTROL	0	6	208.80	18.94	9.072
24	C3	24	CONTROL	0	6	2036.82	371.23	18.226
25	C3	25	CONTROL	0	6	.	.	.
26	C3	26	CONTROL	0	6	217.95	46.48	21.327
27	C3	27	CONTROL	0	6	2243.51	289.97	12.925
28	C3	28	CONTROL	0	6	177.61	172.35	97.040
29	C3	29	CONTROL	0	6	491.07	415.26	84.562
30	C3	30	CONTROL	0	6	2786.38	1293.81	46.433
31	C3	31	CONTROL	0	6	9437.32	1086.34	11.511
32	C3	32	CONTROL	0	6	2538.57	225.46	8.882
33	C3	33	CONTROL	0	6	13840.63	3033.06	21.914
34	C3	34	CONTROL	0	6	67.85	32.83	48.383
35	C3	35	CONTROL	0	6	71.99	48.21	66.959
36	C3	36	CONTROL	0	6	899.73	253.65	28.191
37	C3	37	CONTROL	0	6	198.41	0.00	0.000
38	C3	38	CONTROL	0	6	238.65	15.39	6.449
39	C3	39	CONTROL	0	6	6217.78	4372.15	70.317
40	C3	40	CONTROL	0	6	102.76	79.92	77.772
41	C6	21	CONTROL	0	6	1706.25	400.48	23.471
42	C6	22	CONTROL	0	6	278.77	15.39	5.521
43	C6	23	CONTROL	0	6	1442.72	311.10	21.564
44	C6	24	CONTROL	0	6	627.55	65.07	10.369
45	C6	25	CONTROL	0	6	175.93	67.73	38.501
46	C6	26	CONTROL	0	6	36.73	16.11	43.849

47	C6	27	CONTROL	0	6	2129.90	518.64	24.351
48	C6	28	CONTROL	0	6	60.51	20.16	33.320
49	C6	29	CONTROL	0	6	1756.10	227.47	12.953
50	C6	30	CONTROL	0	6	1697.45	526.60	31.023
51	C6	31	CONTROL	0	6	14.63	3.13	21.411
52	C6	32	CONTROL	0	6	1888.82	361.66	19.147
53	C6	33	CONTROL	0	6	2307.73	238.14	10.319
54	C6	34	CONTROL	0	6	74.49	123.46	165.746
55	C6	35	CONTROL	0	6	45.12	8.36	18.519
56	C6	36	CONTROL	0	6	14815.70	5414.25	36.544
57	C6	37	CONTROL	0	6	1428.39	413.12	28.922
58	C6	38	CONTROL	0	6	3102.61	411.83	13.274
59	C6	39	CONTROL	0	6	3876.90	735.67	18.976
60	C6	40	CONTROL	0	6	3085.45	900.82	29.196
61	E1	1	ATRAZINE	0	6	34.64	4.44	12.830
62	E1	2	ATRAZINE	0	6	1532.07	142.84	9.323
63	E1	3	ATRAZINE	0	6	2127.42	184.66	8.680
64	E1	4	ATRAZINE	0	6	63.66	31.31	49.174
65	E1	5	ATRAZINE	0	6	44.52	33.72	75.747
66	E1	6	ATRAZINE	0	6	.	.	.
67	E3	1	ATRAZINE	0	6	119.73	13.69	11.430
68	E3	2	ATRAZINE	0	6	244.45	99.19	40.576
69	E3	3	ATRAZINE	0	6	154.14	42.81	27.772
70	E3	4	ATRAZINE	0	6	129.56	43.94	33.913
71	E3	5	ATRAZINE	0	6	6.00	0.00	0.000

MEAN FEMALE PLASMA TESTOSTERONE CONCENTRATIONS BY SITE AND ANIMAL NUMBER 99

Obs	Site	Ind_No	TREAT	_TYPE_	_FREQ_	T	STD	CV
72	E3	6	ATRAZINE	0	3	.	.	.
73	E3	7	ATRAZINE	0	6	63.25	42.27	66.832
74	E3	8	ATRAZINE	0	6	110.76	55.10	49.745
75	E3	9	ATRAZINE	0	6	136.19	118.46	86.983
76	E3	10	ATRAZINE	0	6	519.34	783.60	150.883
77	E4	1	ATRAZINE	0	6	501.40	214.98	42.876
78	E4	2	ATRAZINE	0	6	2196.90	244.47	11.128
79	E4	3	ATRAZINE	0	6	663.50	97.44	14.685
80	E4	4	ATRAZINE	0	6	396.55	37.25	9.392
81	E4	5	ATRAZINE	0	6	239.31	50.18	20.969
82	E4	6	ATRAZINE	0	6	4495.01	2233.98	49.699
83	E4	7	ATRAZINE	0	6	371.69	32.59	8.767
84	E4	8	ATRAZINE	0	6	103.61	44.09	42.550
85	E4	9	ATRAZINE	0	6	4473.15	870.89	19.469
86	E4	10	ATRAZINE	0	6	1838.00	170.56	9.280
87	E6	1	ATRAZINE	0	6	72.81	15.26	20.956
88	E6	2	ATRAZINE	0	6	37.52	10.78	28.742
89	E6	3	ATRAZINE	0	6	.	.	.
90	E6	4	ATRAZINE	0	6	99.46	19.78	19.888
91	E6	5	ATRAZINE	0	6	103.05	42.38	41.123
92	E6	6	ATRAZINE	0	6	154.92	30.48	19.675
93	E6	7	ATRAZINE	0	6	29.62	17.58	59.351
94	E6	8	ATRAZINE	0	6	.	.	.
95	E6	9	ATRAZINE	0	6	199.67	112.48	56.334
96	E6	10	ATRAZINE	0	6	70.86	10.91	15.391
97	E8	1	ATRAZINE	0	6	338.68	254.52	75.151
98	E8	2	ATRAZINE	0	6	2491.05	1026.81	41.220
99	E8	3	ATRAZINE	0	6	74.43	22.66	30.452
100	E8	4	ATRAZINE	0	6	186.29	26.58	14.270
101	E8	5	ATRAZINE	0	6	2214.21	1006.46	45.455
102	E8	6	ATRAZINE	0	6	110.45	18.60	16.842
103	E8	7	ATRAZINE	0	6	183.41	15.589	8.4997
104	E8	8	ATRAZINE	0	6	4381.40	601.186	13.7213
105	E8	9	ATRAZINE	0	6	74.89	28.512	38.0707
106	E8	10	ATRAZINE	0	6	34.50	15.136	43.8774
107	E8	11	ATRAZINE	0	6	109.70	24.201	22.0601
108	E8	12	ATRAZINE	0	6	348.47	37.360	10.7213
109	E8	13	ATRAZINE	0	5	.	.	.
110	E8	14	ATRAZINE	0	5	.	.	.
111	E8	15	ATRAZINE	0	5	.	.	.

ANOVA FOR FEMALE PLASMA TESTOSTERONE BETWEEN SITES 101

The ANOVA Procedure

Class Level Information

Class	Levels	Values
Site	8	C1 C3 C6 E1 E3 E4 E6 E8

Number of observations 111

NOTE: Due to missing values, only 102 observations can be used in this analysis.
Dependent Variable: T

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	7	134465197	19209314	1.97	0.0673
Error	94	916631986	9751404		
Corrected Total	101	1051097183			

R-Square	Coeff Var	Root MSE	T Mean
0.127928	168.4578	3122.724	1853.713

Source	DF	Anova SS	Mean Square	F Value	Pr > F
Site	7	134465196.7	19209313.8	1.97	0.0673

Levene's Test for Homogeneity of T Variance
ANOVA of Squared Deviations from Group Means

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Site	7	5.972E15	8.531E14	1.23	0.2963
Error	94	6.54E16	6.958E14		

Bartlett's Test for Homogeneity of T Variance

Source	DF	Chi-Square	Pr > ChiSq
Site	7	114.0	<.0001

Dunnett's t Tests for T

NOTE: This test controls the Type I experimentwise error for comparisons of all treatments against a control.

Alpha	0.05
Error Degrees of Freedom	94
Error Mean Square	9751404
Critical Value of Dunnett's t	2.70019

Comparisons significant at the 0.05 level are indicated by ***.

Site Comparison	Difference Between Means	Simultaneous 95% Confidence Limits	
C3 - C1	-965.2	-3700.9	1770.5
C6 - C1	-1524.7	-4225.9	1176.6
E4 - C1	-2024.3	-5318.5	1269.9
E8 - C1	-2673.3	-5782.4	435.8
E1 - C1	-2791.8	-7029.9	1446.3
E3 - C1	-3387.4	-6799.4	24.6
E6 - C1	-3456.3	-7010.0	97.5

NONPARAMETRIC COMPARISON OF FEMALE PLASMA TESTOSTERONE ACROSS SITES 105

The NPAR1WAY Procedure

Wilcoxon Scores (Rank Sums) for Variable T
Classified by Variable Site

Site	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
C1	19	1173.0	978.50	116.343958	61.736842
C3	19	1145.0	978.50	116.343958	60.263158
C6	20	1122.0	1030.00	118.645129	56.100000
E1	5	171.0	257.50	64.520669	34.200000
E3	9	287.0	463.50	84.759955	31.888889
E4	10	634.0	515.00	88.863191	63.400000
E6	8	178.0	412.00	80.340940	22.250000
E8	12	543.0	618.00	96.280839	45.250000

Kruskal-Wallis Test

Chi-Square	20.0577
DF	7
Pr > Chi-Square	0.0054

Median Scores (Number of Points Above Median) for Variable T
Classified by Variable Site

Site	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
C1	19	12.0	9.50	1.975719	0.631579
C3	19	10.0	9.50	1.975719	0.526316
C6	20	13.0	10.00	2.014797	0.650000
E1	5	2.0	2.50	1.095671	0.400000
E3	9	1.0	4.50	1.439369	0.111111
E4	10	8.0	5.00	1.509049	0.800000
E6	8	0.0	4.00	1.364326	0.000000
E8	12	5.0	6.00	1.635013	0.416667

Median One-Way Analysis

Chi-Square	20.5428
DF	7
Pr > Chi-Square	0.0045

MEAN FEMALE PLASMA TESTOSTERONE CONCENTRATIONS BY TREATMENT 107

Obs	TREAT	_TYPE_	_FREQ_	T	STD	CV
1	ATRAZINE	0	300	724.55	1270.60	175.364
2	CONTROL	0	360	2942.16	4636.87	157.601

ANOVA FOR FEMALE PLASMA TESTOSTERONE ACROSS TREATMENTS 108

The ANOVA Procedure

Class Level Information

Class	Levels	Values
TREAT	2	ATRAZINE CONTROL

Number of observations 111

NOTE: Due to missing values, only 102 observations can be used in this analysis.

Dependent Variable: T

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	1	98659132	98659132	10.36	0.0017
Error	100	952438051	9524381		
Corrected Total	101	1051097183			

R-Square	Coeff Var	Root MSE	T Mean
0.093863	166.4853	3086.160	1853.713

Source	DF	Anova SS	Mean Square	F Value	Pr > F
TREAT	1	98659131.80	98659131.80	10.36	0.0017

Levene's Test for Homogeneity of T Variance
ANOVA of Squared Deviations from Group Means

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
TREAT	1	4.797E15	4.797E15	6.77	0.0107
Error	100	7.081E16	7.081E14		

Bartlett's Test for Homogeneity of T Variance

Source	DF	Chi-Square	Pr > ChiSq
TREAT	1	50.9636	<.0001

Dunnett's t Tests for T

NOTE: This test controls the Type I experimentwise error for comparisons of all treatments against a control.

Alpha	0.05
Error Degrees of Freedom	100
Error Mean Square	9524381
Critical Value of Dunnett's t	1.98398

Comparisons significant at the 0.05 level are indicated by ***.

TREAT Comparison	Difference Between Means	Simultaneous 95% Confidence Limits		
CONTROL - ATRAZINE	1985.8	761.7	3209.9	***

MEAN MALE PLASMA ESTRADIOL CONCENTRATIONS BY SITE AND ANIMAL NUMBER

Obs	Site	Ind_No	TREAT	_TYPE_	_FREQ_	E2	STD	CV
1	C1	1	CONTROL	0	3	15.87	9.19	57.913
2	C1	2	CONTROL	0	3	4492.83	294.58	6.557
3	C1	3	CONTROL	0	3	2404.51	250.12	10.402
4	C1	4	CONTROL	0	3	8.01	2.40	29.951
5	C1	5	CONTROL	0	3	4130.31	228.71	5.537
6	C1	6	CONTROL	0	3	4077.53	326.52	8.008
7	C1	7	CONTROL	0	3	5541.93	1066.63	19.247
8	C1	8	CONTROL	0	3	12.86	4.63	35.962
9	C1	9	CONTROL	0	3	41.91	8.78	20.956
10	C1	10	CONTROL	0	3	31.25	11.07	35.417
11	C1	11	CONTROL	0	3	64380.80	0.00	0.000
12	C1	12	CONTROL	0	3	35.75	17.69	49.488
13	C1	13	CONTROL	0	3	.	.	.
14	C1	14	CONTROL	0	3	98.39	46.18	46.939
15	C1	15	CONTROL	0	3	24.46	3.23	13.187
16	C1	16	CONTROL	0	3	63399.39	0.00	0.000
17	C1	17	CONTROL	0	3	21.41	0.54	2.518
18	C1	18	CONTROL	0	3	13.60	7.82	57.470
19	C1	19	CONTROL	0	3	.	.	.
20	C1	20	CONTROL	0	3	55.10	19.47	35.334
21	C3	1	CONTROL	0	3	.	.	.
22	C3	2	CONTROL	0	3	4683.85	269.53	5.754
23	C3	3	CONTROL	0	3	6290.86	1152.91	18.327
24	C3	4	CONTROL	0	3	48.31	55.54	114.965
25	C3	5	CONTROL	0	3	40.76	9.89	24.262
26	C3	6	CONTROL	0	3	25.63	0.70	2.715
27	C3	7	CONTROL	0	3	23262.42	2510.43	10.792
28	C3	8	CONTROL	0	3	4743.00	257.49	5.429
29	C3	9	CONTROL	0	3	28.66	7.32	25.555
30	C3	10	CONTROL	0	3	6568.09	1080.88	16.456
31	C3	11	CONTROL	0	3	5361.80	893.98	16.673
32	C3	12	CONTROL	0	3	4603.38	113.50	2.466
33	C3	13	CONTROL	0	3	10.13	5.90	58.246
34	C3	14	CONTROL	0	3	62.73	23.13	36.871
35	C3	15	CONTROL	0	3	98.45	.	.
36	C3	16	CONTROL	0	3	315.76	84.04	26.617
37	C3	17	CONTROL	0	3	.	.	.
38	C6	1	CONTROL	0	3	.	.	.
39	C6	2	CONTROL	0	3	433.93	54.03	12.452
40	C6	3	CONTROL	0	3	4619.07	1398.84	30.284
41	C6	4	CONTROL	0	3	29462.37	0.00	0.000
42	C6	5	CONTROL	0	3	7268.35	.	.
43	C6	6	CONTROL	0	3	13.29	6.66	50.074
44	C6	7	CONTROL	0	3	4376.36	307.80	7.033
45	C6	8	CONTROL	0	3	7893.73	619.58	7.849
46	C6	9	CONTROL	0	3	18.34	7.19	39.233

47	C6	10	CONTROL	0	3	5383.19	1515.08	28.145
48	C6	11	CONTROL	0	3	16.80	9.09	54.124
49	C6	12	CONTROL	0	3	26817.92	834.57	3.112
50	C6	13	CONTROL	0	3	3.37	0.00	0.000
51	C6	14	CONTROL	0	3	12815.92	2261.06	17.643
52	C6	15	CONTROL	0	3	10.54	0.00	0.000
53	C6	16	CONTROL	0	3	6065.52	199.53	3.290
54	C6	17	CONTROL	0	3	9.45	0.00	0.000
55	C6	18	CONTROL	0	3	4763.25	1793.75	37.658
56	C6	19	CONTROL	0	3	82865.65	.	.
57	C6	20	CONTROL	0	3	5527.13	292.49	5.292
58	E1	1	ATRAZINE	0	3	193.25	37.43	19.368
59	E1	2	ATRAZINE	0	3	238.75	12.20	5.109
60	E1	3	ATRAZINE	0	3	6.11	0.00	0.000
61	E1	4	ATRAZINE	0	3	106.33	44.92	42.243
62	E1	5	ATRAZINE	0	3	187.66	47.96	25.559
63	E1	6	ATRAZINE	0	3	6.85	0.00	0.000
64	E1	7	ATRAZINE	0	3	8.03	0.00	0.000
65	E1	8	ATRAZINE	0	3	9991.09	2055.96	20.578
66	E3	1	ATRAZINE	0	3	201.97	70.70	35.004
67	E3	2	ATRAZINE	0	3	182.84	7.29	3.987
68	E3	3	ATRAZINE	0	3	507.43	53.75	10.592
69	E3	4	ATRAZINE	0	3	9399.57	1177.61	12.528
70	E3	5	ATRAZINE	0	3	244.15	39.68	16.254
71	E3	6	ATRAZINE	0	3	263.68	31.09	11.790

MEAN MALE PLASMA ESTRADIOL CONCENTRATIONS BY SITE AND ANIMAL NUMBER

Obs	Site	Ind_No	TREAT	_TYPE_	_FREQ_	E2	STD	CV
72	E3	7	ATRAZINE	0	3	7777.23	759.22	9.762
73	E3	8	ATRAZINE	0	3	182.68	27.28	14.934
74	E3	9	ATRAZINE	0	3	5.14	0.00	0.000
75	E3	10	ATRAZINE	0	3	149.09	13.68	9.179
76	E4	1	ATRAZINE	0	3	7943.10	3964.57	49.912
77	E4	2	ATRAZINE	0	3	150.47	18.66	12.403
78	E4	3	ATRAZINE	0	3	79.94	52.95	66.242
79	E4	4	ATRAZINE	0	3	36.94	21.52	58.246
80	E4	5	ATRAZINE	0	3	3410.68	436.81	12.807
81	E4	6	ATRAZINE	0	3	3640.90	352.58	9.684
82	E4	7	ATRAZINE	0	3	.	.	.
83	E4	8	ATRAZINE	0	3	6524.77	286.56	4.392
84	E4	9	ATRAZINE	0	3	20742.65	2690.87	12.973
85	E4	10	ATRAZINE	0	3	23.56	24.36	103.364
86	E6	1	ATRAZINE	0	3	108.45	39.44	36.370
87	E6	2	ATRAZINE	0	3	29.56	18.42	62.328
88	E6	3	ATRAZINE	0	3	103.20	11.59	11.230
89	E6	4	ATRAZINE	0	3	129.57	48.82	37.675
90	E6	5	ATRAZINE	0	3	166.00	15.83	9.538
91	E6	6	ATRAZINE	0	3	63.51	12.19	19.192
92	E6	7	ATRAZINE	0	3	180.53	104.00	57.608
93	E6	8	ATRAZINE	0	3	6032.94	293.69	4.868
94	E6	9	ATRAZINE	0	3	131.07	19.68	15.011
95	E6	10	ATRAZINE	0	3	.	.	.
96	E8	1	ATRAZINE	0	3	86.70	21.36	24.632
97	E8	2	ATRAZINE	0	3	192.36	28.20	14.662
98	E8	3	ATRAZINE	0	3	2861.36	166.52	5.820
99	E8	4	ATRAZINE	0	3	.	.	.
100	E8	5	ATRAZINE	0	3	134.59	26.26	19.513
101	E8	6	ATRAZINE	0	3	129.23	58.11	44.970

ANOVA FOR MALE PLASMA ESTRADIOL BETWEEN SITES 114

The ANOVA Procedure

Class Level Information

Class	Levels	Values
Site	8	C1 C3 C6 E1 E3 E4 E6 E8

Number of observations 101

NOTE: Due to missing values, only 93 observations can be used in this analysis.
Dependent Variable: E2

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	7	1231861396	175980199	1.00	0.4368
Error	85	14951054355	175894757		
Corrected Total	92	16182915750			

R-Square	Coeff Var	Root MSE	E2 Mean
0.076121	253.8687	13262.53	5224.170

Source	DF	Anova SS	Mean Square	F Value	Pr > F
Site	7	1231861396	175980199	1.00	0.4368

Levene's Test for Homogeneity of E2 Variance
ANOVA of Squared Deviations from Group Means

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Site	7	2.821E18	4.031E17	0.82	0.5770
Error	85	4.201E19	4.943E17		

Bartlett's Test for Homogeneity of E2 Variance

Source	DF	Chi-Square	Pr > ChiSq
Site	7	92.5314	<.0001

Dunnett's t Tests for E2

NOTE: This test controls the Type I experimentwise error for comparisons of all treatments against a control.

Alpha	0.05
Error Degrees of Freedom	85
Error Mean Square	1.7589E8
Critical Value of Dunnett's t	2.70761

Comparisons significant at the 0.05 level are indicated by ***.

Site Comparison	Difference Between Means	Simultaneous 95% Confidence Limits	
C6 - C1	2174	-9637	13986
E4 - C1	-3538	-18198	11122
C3 - C1	-4523	-17077	8031
E3 - C1	-6375	-20537	7788
E1 - C1	-6924	-22182	8335
E6 - C1	-7494	-22154	7166
E8 - C1	-7585	-25738	10568

NONPARAMETRIC COMPARISON OF MALE PLASMA ESTRADIOL BETWEEN SITES 118

The NPAR1WAY Procedure

Wilcoxon Scores (Rank Sums) for Variable E2
Classified by Variable Site

Site	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
C1	18	711.0	846.0	102.834819	39.500000
C3	15	735.0	705.0	95.734006	49.000000
C6	19	1055.0	893.0	104.946018	55.526316
E1	8	284.0	376.0	72.984017	35.500000
E3	10	517.0	470.0	80.632913	51.700000
E4	9	484.0	423.0	76.954532	53.777778
E6	9	366.0	423.0	76.954532	40.666667
E8	5	219.0	235.0	58.708319	43.800000

Kruskal-Wallis Test

Chi-Square	6.2571
DF	7
Pr > Chi-Square	0.5101

Median Scores (Number of Points Above Median) for Variable E2
Classified by Variable Site

Site	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
C1	18	7.0	8.903226	1.915216	0.388889
C3	15	8.0	7.419355	1.782969	0.533333
C6	19	13.0	9.397849	1.954536	0.684211
E1	8	4.0	3.956989	1.359269	0.500000
E3	10	6.0	4.946237	1.501724	0.600000
E4	9	5.0	4.451613	1.433217	0.555556
E6	9	1.0	4.451613	1.433217	0.111111
E8	5	2.0	2.473118	1.093396	0.400000

Median One-Way Analysis

Chi-Square	9.5763
DF	7
Pr > Chi-Square	0.2139

MEAN MALE PLASMA ESTRADIOL CONCENTRATIONS BY TREATMENT 120

Obs	TREAT	_TYPE_	_FREQ_	E2	STD	CV
1	ATRAZINE	0	132	2013.51	4174.05	207.302
2	CONTROL	0	171	6878.78	15084.70	219.293

ANOVA FOR MALE PLASMA ESTRADIOL ACROSS TREATMENTS 121

The ANOVA Procedure

Class Level Information

Class	Levels	Values
TREAT	2	ATRAZINE CONTROL

Number of observations 101

NOTE: Due to missing values, only 93 observations can be used in this analysis.

ANOVA FOR MALE PLASMA ESTRADIOL ACROSS TREATMENTS 122

The ANOVA Procedure

Dependent Variable: E2

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	1	755878968	755878968	4.46	0.0375
Error	91	15427036782	169527877		
Corrected Total	92	16182915750			

R-Square	Coeff Var	Root MSE	E2 Mean
0.046708	249.2317	13020.29	5224.170

Source	DF	Anova SS	Mean Square	F Value	Pr > F
TREAT	1	755878968.2	755878968.2	4.46	0.0375

Levene's Test for Homogeneity of E2 Variance
ANOVA of Squared Deviations from Group Means

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
TREAT	1	1.63E18	1.63E18	3.08	0.0826
Error	91	4.814E19	5.291E17		

Bartlett's Test for Homogeneity of E2 Variance

Source	DF	Chi-Square	Pr > ChiSq
TREAT	1	63.5686	<.0001

Dunnett's t Tests for E2

NOTE: This test controls the Type I experimentwise error for comparisons of all treatments against a control.

Alpha	0.05
Error Degrees of Freedom	91
Error Mean Square	1.6953E8
Critical Value of Dunnett's t	1.98639

Comparisons significant at the 0.05 level are indicated by ***.

TREAT Comparison	Difference Between Means	Simultaneous 95% Confidence Limits		
CONTROL - ATRAZINE	5742	340	11144	***

MEAN MALE PLASMA TESTOSTERONE CONCENTRATIONS BY SITE AND ANIMAL NUMBER 125

Obs	Site	Ind_No	TREAT	_TYPE_	_FREQ_	T	STD	CV
1	C1	1	CONTROL	0	6	139.03	139.95	100.663
2	C1	2	CONTROL	0	6	2737.00	242.96	8.877
3	C1	3	CONTROL	0	6	1674.80	769.50	45.946
4	C1	4	CONTROL	0	6	83.59	28.35	33.911
5	C1	5	CONTROL	0	6	2696.11	388.14	14.396
6	C1	6	CONTROL	0	6	2581.18	188.89	7.318
7	C1	7	CONTROL	0	6	2896.29	226.63	7.825
8	C1	8	CONTROL	0	6	194.78	39.95	20.511
9	C1	9	CONTROL	0	6	144.59	27.16	18.786
10	C1	10	CONTROL	0	6	82.04	6.21	7.566
11	C1	11	CONTROL	0	6	32190.40	0.00	0.000
12	C1	12	CONTROL	0	6	113.76	24.18	21.255
13	C1	13	CONTROL	0	6	.	.	.
14	C1	14	CONTROL	0	6	59.60	13.25	22.232
15	C1	15	CONTROL	0	6	57.70	18.65	32.317
16	C1	16	CONTROL	0	6	21112.00	0.00	0.000
17	C1	17	CONTROL	0	6	31.36	5.77	18.406
18	C1	18	CONTROL	0	6	88.85	15.25	17.161
19	C1	19	CONTROL	0	6	68.54	4.30	6.279
20	C1	20	CONTROL	0	6	161.21	35.78	22.196
21	C3	1	CONTROL	0	6	.	.	.
22	C3	2	CONTROL	0	6	2208.90	518.72	23.483
23	C3	3	CONTROL	0	6	9935.35	1378.10	13.871
24	C3	4	CONTROL	0	6	592.11	68.26	11.528
25	C3	5	CONTROL	0	6	265.97	175.53	65.994
26	C3	6	CONTROL	0	6	654.53	239.70	36.622
27	C3	7	CONTROL	0	6	8145.75	547.31	6.719
28	C3	8	CONTROL	0	6	2755.58	1362.81	49.456
29	C3	9	CONTROL	0	6	.	.	.
30	C3	10	CONTROL	0	6	2611.80	296.82	11.364
31	C3	11	CONTROL	0	6	2376.55	142.75	6.006
32	C3	12	CONTROL	0	6	2372.41	421.51	17.767
33	C3	13	CONTROL	0	6	159.89	97.48	60.967
34	C3	14	CONTROL	0	6	3556.26	417.34	11.735
35	C3	15	CONTROL	0	6	221.63	28.98	13.076
36	C3	16	CONTROL	0	6	190.61	137.99	72.396
37	C3	17	CONTROL	0	6	.	.	.
38	C6	1	CONTROL	0	6	79.25	22.98	28.991
39	C6	2	CONTROL	0	6	1217.66	856.84	70.368
40	C6	3	CONTROL	0	6	3018.72	506.00	16.762
41	C6	4	CONTROL	0	6	12570.29	909.41	7.235
42	C6	5	CONTROL	0	6	3905.44	1376.31	35.241
43	C6	6	CONTROL	0	6	224.60	40.01	17.816
44	C6	7	CONTROL	0	6	1857.56	339.98	18.302
45	C6	8	CONTROL	0	6	4493.02	1951.16	43.426
46	C6	9	CONTROL	0	6	685.27	98.23	14.334

47	C6	10	CONTROL	0	6	2264.66	220.37	9.731
48	C6	11	CONTROL	0	6	602.58	86.20	14.305
49	C6	12	CONTROL	0	6	1928.61	329.99	17.110
50	C6	13	CONTROL	0	6	204.25	37.57	18.393
51	C6	14	CONTROL	0	6	5314.58	1069.39	20.122
52	C6	15	CONTROL	0	6	1034.23	35.23	3.4068
53	C6	16	CONTROL	0	6	3573.74	1077.58	30.1527
54	C6	17	CONTROL	0	6	9867.56	773.75	7.8413
55	C6	18	CONTROL	0	6	1494.39	143.13	9.5777
56	C6	19	CONTROL	0	6	7387.18	1162.12	15.7315
57	C6	20	CONTROL	0	6	1603.54	282.26	17.6021
58	E1	1	ATRAZINE	0	6	831.37	85.38	10.2698
59	E1	2	ATRAZINE	0	6	484.80	16.27	3.3550
60	E1	3	ATRAZINE	0	6	763.44	23.01	3.0134
61	E1	4	ATRAZINE	0	6	245.16	33.41	13.6296
62	E1	5	ATRAZINE	0	6	192.02	56.66	29.5075
63	E1	6	ATRAZINE	0	6	146.08	12.43	8.5100
64	E1	7	ATRAZINE	0	6	56.13	6.37	11.3552
65	E1	8	ATRAZINE	0	6	8262.43	1120.15	13.5572
66	E3	1	ATRAZINE	0	6	598.56	46.73	7.8074
67	E3	2	ATRAZINE	0	6	324.72	35.74	11.0078
68	E3	3	ATRAZINE	0	6	595.42	198.93	33.4092
69	E3	4	ATRAZINE	0	6	5124.19	260.81	5.0898
70	E3	5	ATRAZINE	0	6	440.39	59.79	13.5763

MEAN MALE PLASMA TESTOSTERONE CONCENTRATIONS BY SITE AND ANIMAL NUMBER

Obs	Site	Ind_No	TREAT	_TYPE_	_FREQ_	T	STD	CV
71	E3	6	ATRAZINE	0	6	255.93	28.83	11.2640
72	E3	7	ATRAZINE	0	6	3936.60	660.75	16.7849
73	E3	8	ATRAZINE	0	6	138.52	48.12	34.7396
74	E3	9	ATRAZINE	0	6	250.10	32.27	12.9048
75	E3	10	ATRAZINE	0	6	252.42	39.72	15.7351
76	E4	1	ATRAZINE	0	6	5144.47	203.92	3.9639
77	E4	2	ATRAZINE	0	6	1060.29	112.53	10.6135
78	E4	3	ATRAZINE	0	6	2872.23	1444.02	50.2754
79	E4	4	ATRAZINE	0	6	1546.88	560.44	36.2305
80	E4	5	ATRAZINE	0	6	1638.72	291.68	17.7991
81	E4	6	ATRAZINE	0	6	3915.86	898.21	22.9377
82	E4	7	ATRAZINE	0	6	.	.	.
83	E4	8	ATRAZINE	0	6	5112.10	796.32	15.5771
84	E4	9	ATRAZINE	0	6	15218.92	2038.78	13.3963
85	E4	10	ATRAZINE	0	6	4992.90	3455.19	69.2020
86	E6	1	ATRAZINE	0	6	185.87	12.22	6.5729
87	E6	2	ATRAZINE	0	6	86.22	22.24	25.7912
88	E6	3	ATRAZINE	0	6	685.96	218.27	31.8191
89	E6	4	ATRAZINE	0	6	124.22	6.12	4.9292
90	E6	5	ATRAZINE	0	6	179.40	42.24	23.5432
91	E6	6	ATRAZINE	0	6	63.85	8.17	12.7968
92	E6	7	ATRAZINE	0	6	92.07	3.03	3.2897
93	E6	8	ATRAZINE	0	6	2759.97	218.01	7.8989
94	E6	9	ATRAZINE	0	6	47.38	18.61	39.2792
95	E6	10	ATRAZINE	0	6	.	.	.
96	E8	1	ATRAZINE	0	6	241.58	54.17	22.4250
97	E8	2	ATRAZINE	0	6	503.73	56.20	11.1575
98	E8	3	ATRAZINE	0	6	1441.63	307.45	21.3265
99	E8	4	ATRAZINE	0	6	.	.	.
100	E8	5	ATRAZINE	0	6	876.52	152.11	17.3543
101	E8	6	ATRAZINE	0	6	81.07	20.93	25.8156

ANOVA FOR MALE PLASMA TESTOSTERONE BETWEEN SITES 127

The ANOVA Procedure

Class Level Information

Class	Levels	Values
Site	8	C1 C3 C6 E1 E3 E4 E6 E8

Number of observations 101

NOTE: Due to missing values, only 94 observations can be used in this analysis.

Dependent Variable: T

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	7	151109859	21587123	1.00	0.4339
Error	86	1848208528	21490797		
Corrected Total	93	1999318387			

R-Square	Coeff Var	Root MSE	T Mean
0.075581	182.8974	4635.817	2534.654

Source	DF	Anova SS	Mean Square	F Value	Pr > F
Site	7	151109858.7	21587122.7	1.00	0.4339

Levene's Test for Homogeneity of T Variance
ANOVA of Squared Deviations from Group Means

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Site	7	5.575E16	7.964E15	0.96	0.4620
Error	86	7.098E17	8.254E15		

Bartlett's Test for Homogeneity of T Variance

Source	DF	Chi-Square	Pr > ChiSq
Site	7	68.1321	<.0001

Dunnett's t Tests for T

NOTE: This test controls the Type I experimentwise error for comparisons of all treatments against a control.

Alpha 0.05

Error Degrees of Freedom	86
Error Mean Square	21490797
Critical Value of Dunnett's t	2.70970

Comparisons significant at the 0.05 level are indicated by ***.

Site Comparison	Difference Between Means	Simultaneous 95% Confidence Limits	
E4 - C1	1079	-4004	6162
C6 - C1	-366	-4390	3658
C3 - C1	-957	-5382	3467
E1 - C1	-2160	-7454	3135
E3 - C1	-2341	-7248	2567
E8 - C1	-2903	-9217	3410
E6 - C1	-3063	-8146	2020

NONPARAMETRIC COMPARISON OF MALE PLASMA TESTOSTERONE ACROSS SITES 131

The NPAR1WAY Procedure

Wilcoxon Scores (Rank Sums) for Variable T
Classified by Variable Site

Site	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
C1	19	674.0	902.50	106.213229	35.473684
C3	14	777.0	665.00	94.162979	55.500000
C6	20	1214.0	950.00	108.243553	60.700000
E1	8	305.0	380.00	73.801536	38.125000
E3	10	441.0	475.00	81.547532	44.100000
E4	9	655.0	427.50	77.821912	72.777778
E6	9	215.0	427.50	77.821912	23.888889
E8	5	184.0	237.50	59.354163	36.800000

Kruskal-Wallis Test

Chi-Square	25.9190
DF	7
Pr > Chi-Square	0.0005

Median Scores (Number of Points Above Median) for Variable T
Classified by Variable Site

Site	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
C1	19	7.0	9.50	1.957203	0.368421
C3	14	8.0	7.00	1.735152	0.571429
C6	20	15.0	10.00	1.994616	0.750000
E1	8	3.0	4.00	1.359949	0.375000
E3	10	2.0	5.00	1.502686	0.200000
E4	9	9.0	4.50	1.434033	1.000000
E6	9	1.0	4.50	1.434033	0.111111
E8	5	2.0	2.50	1.093726	0.400000

Median One-Way Analysis

Chi-Square	25.0763
DF	7
Pr > Chi-Square	0.0007

MEAN MALE PLASMA TESTOSTERONE CONCENTRATIONS BY TREATMENT 133

Obs	TREAT	_TYPE_	_FREQ_	T	STD	CV
1	ATRAZINE	0	264	1750.49	2943.22	168.137
2	CONTROL	0	342	3113.39	5255.50	168.803

ANOVA FOR MALE PLASMA TESTOSTERONE ACROSS TREATMENTS 134

The ANOVA Procedure

Class Level Information

Class	Levels	Values
TREAT	2	ATRAZINE CONTROL

Number of observations 101

NOTE: Due to missing values, only 94 observations can be used in this analysis.

Dependent Variable: T

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	1	44714480	44714480	2.10	0.1503
Error	92	1954603906	21245695		
Corrected Total	93	1999318387			

R-Square	Coeff Var	Root MSE	T Mean
0.022365	181.8515	4609.305	2534.654

Source	DF	Anova SS	Mean Square	F Value	Pr > F
TREAT	1	44714480.50	44714480.50	2.10	0.1503

Levene's Test for Homogeneity of T Variance
ANOVA of Squared Deviations from Group Means

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
TREAT	1	1.147E16	1.147E16	1.30	0.2579
Error	92	8.145E17	8.853E15		

Bartlett's Test for Homogeneity of T Variance

Source	DF	Chi-Square	Pr > ChiSq
TREAT	1	16.9361	<.0001

Dunnett's t Tests for T

NOTE: This test controls the Type I experimentwise error for comparisons of all treatments against a control.

Alpha	0.05
Error Degrees of Freedom	92
Error Mean Square	21245695
Critical Value of Dunnett's t	1.98609

Comparisons significant at the 0.05 level are indicated by ***.

TREAT Comparison	Difference Between Means	Simultaneous 95% Confidence Limits	
CONTROL - ATRAZINE	1390.8	-513.2	3294.8

MEAN FEMALE AROMATASE ACTIVITY BY SITE AND ANIMAL NUMBER 138

Obs	Site	Ind_No	TREAT	_TYPE_	_FREQ_	A	STD	CV
1	C1	21	CONTROL	0	2	153.70	5.5665	3.6218
2	C1	22	CONTROL	0	2	26.59	1.0643	4.0021
3	C1	23	CONTROL	0	2	80.68	7.1499	8.8622
4	C1	24	CONTROL	0	2	160.21	13.3182	8.3131
5	C1	25	CONTROL	0	2	4.54	0.3710	8.1696
6	C1	26	CONTROL	0	2	138.02	3.7682	2.7301
7	C1	27	CONTROL	0	2	53.28	3.2616	6.1219
8	C1	28	CONTROL	0	2	28.71	1.6017	5.5786
9	C1	29	CONTROL	0	2	61.51	6.6017	10.7327
10	C1	30	CONTROL	0	2	31.80	3.7195	11.6980
11	C1	31	CONTROL	0	2	82.95	3.5850	4.3219
12	C1	32	CONTROL	0	2	13.51	0.1155	0.8547
13	C1	33	CONTROL	0	2	93.41	6.4528	6.9083
14	C1	34	CONTROL	0	2	8.99	0.0119	0.1329
15	C1	35	CONTROL	0	2	.	.	.
16	C1	36	CONTROL	0	2	84.56	1.5973	1.8888
17	C1	37	CONTROL	0	2	11.18	0.2770	2.4780
18	C1	38	CONTROL	0	2	52.10	4.7007	9.0231
19	C1	39	CONTROL	0	2	.	.	.
20	C1	40	CONTROL	0	2	68.07	5.0388	7.4027
21	C3	21	CONTROL	0	2	185.19	0.1718	0.0928
22	C3	22	CONTROL	0	2	50.45	3.9401	7.8098
23	C3	23	CONTROL	0	2	725.37	3.2272	0.4449
24	C3	24	CONTROL	0	2	328.68	8.1464	2.4785
25	C3	25	CONTROL	0	2	1409.86	50.6053	3.5894
26	C3	26	CONTROL	0	2	113.26	2.0057	1.7709
27	C3	27	CONTROL	0	2	59.76	2.5647	4.2918
28	C3	28	CONTROL	0	2	308.24	0.6218	0.2017
29	C3	29	CONTROL	0	2	120.86	2.6017	2.1527
30	C3	30	CONTROL	0	2	102.66	0.3044	0.2965
31	C3	31	CONTROL	0	2	790.03	4.1639	0.5271
32	C3	32	CONTROL	0	2	357.96	8.6876	2.4269
33	C3	33	CONTROL	0	2	886.19	0.6158	0.0695
34	C3	34	CONTROL	0	2	130.78	1.5195	1.1619
35	C3	35	CONTROL	0	2	47.21	1.4813	3.1376
36	C3	36	CONTROL	0	2	182.98	1.6464	0.8998
37	C3	37	CONTROL	0	2	70.96	1.4720	2.0742
38	C3	38	CONTROL	0	2	215.63	4.4122	2.0462
39	C3	39	CONTROL	0	2	279.03	1.4497	0.5195
40	C3	40	CONTROL	0	2	194.26	5.8231	2.9975
41	C6	21	CONTROL	0	2	33.45	0.3585	1.0717
42	C6	22	CONTROL	0	2	29.55	0.4214	1.4258
43	C6	23	CONTROL	0	2	4.03	0.3213	7.9645
44	C6	24	CONTROL	0	2	.	.	.
45	C6	25	CONTROL	0	2	19.69	0.7304	3.7097

46	C6	26	CONTROL	0	2	3.07	0.8702	28.3025
47	C6	27	CONTROL	0	2	0.23	0.0352	15.1253
48	C6	28	CONTROL	0	2	24.29	0.4593	1.8907
49	C6	29	CONTROL	0	2	.	.	.
50	C6	30	CONTROL	0	2	31.64	4.3236	13.6663
51	C6	31	CONTROL	0	2	.	.	.
52	C6	32	CONTROL	0	2	5.482	0.2420	4.4148
53	C6	33	CONTROL	0	2	4.245	0.5198	12.2429
54	C6	34	CONTROL	0	2	23.058	0.9027	3.9149
55	C6	35	CONTROL	0	2	24.524	0.0634	0.2584
56	C6	36	CONTROL	0	2	.	.	.
57	C6	37	CONTROL	0	2	22.756	0.0416	0.1830
58	C6	38	CONTROL	0	2	2.335	0.7771	33.2828
59	C6	39	CONTROL	0	2	18.042	3.0613	16.9672
60	C6	40	CONTROL	0	2	2.070	0.1799	8.6880
61	E1	1	ATRAZINE	0	2	78.142	0.2266	0.2899
62	E1	2	ATRAZINE	0	2	465.218	9.6807	2.0809
63	E1	3	ATRAZINE	0	2	34.272	1.5139	4.4172
64	E1	4	ATRAZINE	0	2	345.492	15.7471	4.5579
65	E1	5	ATRAZINE	0	2	153.642	1.7230	1.1214
66	E1	6	ATRAZINE	0	2	39.003	0.7964	2.0418
67	E3	1	ATRAZINE	0	2	25.565	0.8305	3.2488
68	E3	2	ATRAZINE	0	2	20.220	0.3865	1.9116
69	E3	3	ATRAZINE	0	2	149.341	2.2374	1.4982

MEAN FEMALE AROMATASE ACTIVITY BY SITE AND ANIMAL NUMBER 139

Obs	Site	Ind_No	TREAT	_TYPE_	_FREQ_	A	STD	CV
70	E3	4	ATRAZINE	0	2	129.350	0.7513	0.5808
71	E3	5	ATRAZINE	0	2	41.427	0.7490	1.8080
72	E3	6	ATRAZINE	0	2	19.066	0.6227	3.2662
73	E3	7	ATRAZINE	0	2	12.951	2.5439	19.6431
74	E3	8	ATRAZINE	0	2	91.475	2.0786	2.2723
75	E3	9	ATRAZINE	0	2	21.132	0.1224	0.5793
76	E3	10	ATRAZINE	0	2	31.900	0.2137	0.6699
77	E4	1	ATRAZINE	0	2	48.127	4.5793	9.5151
78	E4	2	ATRAZINE	0	2	39.544	3.2288	8.1650
79	E4	3	ATRAZINE	0	2	71.724	0.0110	0.0153
80	E4	4	ATRAZINE	0	2	17.726	2.3697	13.3684
81	E4	5	ATRAZINE	0	2	.	.	.
82	E4	6	ATRAZINE	0	2	78.712	2.6865	3.4131
83	E4	7	ATRAZINE	0	2	35.272	2.6168	7.4189
84	E4	8	ATRAZINE	0	2	137.632	3.3057	2.4019
85	E4	9	ATRAZINE	0	2	205.137	6.1020	2.9746
86	E4	10	ATRAZINE	0	2	143.463	8.7359	6.0893
87	E6	1	ATRAZINE	0	2	84.509	0.1741	0.2060
88	E6	2	ATRAZINE	0	2	61.436	1.5234	2.4797
89	E6	3	ATRAZINE	0	2	52.416	0.7188	1.3713
90	E6	4	ATRAZINE	0	2	56.748	1.8847	3.3211
91	E6	5	ATRAZINE	0	2	74.332	2.6188	3.5231
92	E6	6	ATRAZINE	0	2	105.314	1.3007	1.2351
93	E6	7	ATRAZINE	0	2	44.214	1.0374	2.3463
94	E6	8	ATRAZINE	0	2	162.100	6.6676	4.1133
95	E6	9	ATRAZINE	0	2	26.301	0.1825	0.6937
96	E6	10	ATRAZINE	0	2	247.262	5.6848	2.2991
97	E8	1	ATRAZINE	0	2	66.779	3.0958	4.6359
98	E8	2	ATRAZINE	0	2	41.074	2.6246	6.3900
99	E8	3	ATRAZINE	0	2	117.380	5.7140	4.8679
100	E8	4	ATRAZINE	0	2	288.086	17.8714	6.2035
101	E8	5	ATRAZINE	0	2	838.400	22.9345	2.7355
102	E8	6	ATRAZINE	0	2	775.106	55.8228	7.2020
103	E8	7	ATRAZINE	0	2	142.010	8.6639	6.10087
104	E8	8	ATRAZINE	0	2	616.996	29.9642	4.85647
105	E8	9	ATRAZINE	0	2	183.710	12.7623	6.94694
106	E8	10	ATRAZINE	0	2	436.498	28.6119	6.55487
107	E8	11	ATRAZINE	0	2	355.159	22.3086	6.28129
108	E8	12	ATRAZINE	0	2	407.640	12.3229	3.02299
109	E8	13	ATRAZINE	0	2	.	.	.
110	E8	14	ATRAZINE	0	2	.	.	.
111	E8	15	ATRAZINE	0	2	.	.	.

The ANOVA Procedure

Class Level Information

Class	Levels	Values
Site	8	C1 C3 C6 E1 E3 E4 E6 E8

Number of observations 111

NOTE: Due to missing values, only 101 observations can be used in this analysis.

Dependent Variable: A

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	7	1731846.446	247406.635	6.57	<.0001
Error	93	3504430.360	37682.047		
Corrected Total	100	5236276.806			

R-Square	Coeff Var	Root MSE	A Mean
0.330740	125.8355	194.1186	154.2639

Source	DF	Anova SS	Mean Square	F Value	Pr > F
Site	7	1731846.446	247406.635	6.57	<.0001

Levene's Test for Homogeneity of A Variance
ANOVA of Squared Deviations from Group Means

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Site	7	2.256E11	3.222E10	2.23	0.0385
Error	93	1.344E12	1.445E10		

Bartlett's Test for Homogeneity of A Variance

Source	DF	Chi-Square	Pr > ChiSq
Site	7	156.5	<.0001

Dunnett's t Tests for A

NOTE: This test controls the Type I experimentwise error for comparisons of all treatments against a control.

Alpha	0.05
Error Degrees of Freedom	93
Error Mean Square	37682.05
Critical Value of Dunnett's t	2.69659

Comparisons significant at the 0.05 level are indicated by ***.

Site Comparison	Difference Between Means	Simultaneous 95% Confidence Limits		
E8 - C1	291.64	96.56	486.72	***
C3 - C1	263.87	93.80	433.94	***
E1 - C1	121.86	-124.90	368.62	
E6 - C1	27.36	-179.09	233.82	
E4 - C1	22.27	-191.43	235.97	
E3 - C1	-9.86	-216.31	196.60	
C6 - C1	-48.57	-228.43	131.29	

The NPAR1WAY Procedure

Wilcoxon Scores (Rank Sums) for Variable A
Classified by Variable Site

Site	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
C1	18	756.0	918.0	112.689840	42.000000
C3	20	1505.0	1020.0	117.345643	75.250000
C6	16	227.0	816.0	107.517440	14.187500
E1	6	377.0	306.0	69.606034	62.833333
E3	10	351.0	510.0	87.948849	35.100000
E4	9	451.0	459.0	83.892789	50.111111
E6	10	532.0	510.0	87.948849	53.200000
E8	12	952.0	612.0	95.278539	79.333333

Kruskal-Wallis Test

Chi-Square	55.8636
DF	7
Pr > Chi-Square	<.0001

Median Scores (Number of Points Above Median) for Variable A
Classified by Variable Site

Site	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
C1	18	7.0	8.910891	1.932520	0.388889
C3	20	16.0	9.900990	2.012363	0.800000
C6	16	0.0	7.920792	1.843819	0.000000
E1	6	4.0	2.970297	1.193675	0.666667
E3	10	3.0	4.950495	1.508236	0.300000
E4	9	5.0	4.455446	1.438679	0.555556
E6	10	5.0	4.950495	1.508236	0.500000
E8	12	10.0	5.940594	1.633933	0.833333

Median One-Way Analysis

Chi-Square	31.4784
DF	7
Pr > Chi-Square	<.0001

MEAN FEMALE AROMATASE ACTIVITY BY TREATMENT 147

Obs	TREAT	_TYPE_	_FREQ_	A	STD	CV
1	ATRAZINE	0	102	162.106	193.158	119.155
2	CONTROL	0	120	147.438	255.819	173.510

ANOVA FOR FEMALE AROMATASE ACTIVITY ACROSS TREATMENTS 148

The ANOVA Procedure

Class Level Information

Class	Levels	Values
TREAT	2	ATRAZINE CONTROL

Number of observations 111

NOTE: Due to missing values, only 101 observations can be used in this analysis.

Dependent Variable: A

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	1	5406.778	5406.778	0.10	0.7497
Error	99	5230870.029	52837.071		
Corrected Total	100	5236276.806			

R-Square	Coeff Var	Root MSE	A Mean
0.001033	149.0065	229.8632	154.2639

Source	DF	Anova SS	Mean Square	F Value	Pr > F
TREAT	1	5406.777677	5406.777677	0.10	0.7497

Levene's Test for Homogeneity of A Variance
ANOVA of Squared Deviations from Group Means

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
TREAT	1	1.966E10	1.966E10	0.60	0.4416
Error	99	3.26E12	3.293E10		

Bartlett's Test for Homogeneity of A Variance

Source	DF	Chi-Square	Pr > ChiSq

TREAT	1	3.7531	0.0527

Dunnett's t Tests for A

NOTE: This test controls the Type I experimentwise error for comparisons of all treatments against a control.

Alpha	0.05
Error Degrees of Freedom	99
Error Mean Square	52837.07
Critical Value of Dunnett's t	1.98422

Comparisons significant at the 0.05 level are indicated by ***.

TREAT Comparison	Difference Between Means	Simultaneous 95% Confidence Limits	
CONTROL - ATRAZINE	-14.67	-105.65	76.32

MEAN MALE AROMATASE ACTIVITY BY SITE AND ANIMAL NUMBER 152

Obs	Site	Ind_No	TREAT	_TYPE_	_FREQ_	A	STD	CV
1	C1	1	CONTROL	0	2	.	.	.
2	C1	2	CONTROL	0	2	.	.	.
3	C1	3	CONTROL	0	2	.	.	.
4	C1	4	CONTROL	0	2	.	.	.
5	C1	5	CONTROL	0	2	.	.	.
6	C1	6	CONTROL	0	2	.	.	.
7	C1	7	CONTROL	0	2	.	.	.
8	C1	8	CONTROL	0	2	.	.	.
9	C1	9	CONTROL	0	2	.	.	.
10	C1	10	CONTROL	0	2	.	.	.
11	C1	11	CONTROL	0	2	.	.	.
12	C1	12	CONTROL	0	2	.	.	.
13	C1	13	CONTROL	0	2	.	.	.
14	C1	14	CONTROL	0	2	.	.	.
15	C1	15	CONTROL	0	2	.	.	.
16	C1	16	CONTROL	0	2	.	.	.
17	C1	17	CONTROL	0	2	.	.	.
18	C1	18	CONTROL	0	2	.	.	.
19	C1	19	CONTROL	0	2	.	.	.
20	C1	20	CONTROL	0	2	.	.	.
21	C3	1	CONTROL	0	2	.	.	.
22	C3	2	CONTROL	0	2	.	.	.
23	C3	3	CONTROL	0	2	21.1445	.	.
24	C3	4	CONTROL	0	2	22.9172	13.5041	58.926
25	C3	5	CONTROL	0	2	20.3058	6.1536	30.305
26	C3	6	CONTROL	0	2	24.3767	22.0849	90.598
27	C3	7	CONTROL	0	2	22.2198	2.3277	10.476
28	C3	8	CONTROL	0	2	16.0041	12.6224	78.870
29	C3	9	CONTROL	0	2	6.8434	.	.
30	C3	10	CONTROL	0	2	22.5209	11.1054	49.311
31	C3	11	CONTROL	0	2	.	.	.
32	C3	12	CONTROL	0	2	5.9473	0.8273	13.910
33	C3	13	CONTROL	0	2	10.6430	12.5429	117.851
34	C3	14	CONTROL	0	2	52.5030	44.8708	85.463
35	C3	15	CONTROL	0	2	.	.	.
36	C3	16	CONTROL	0	2	4.2133	.	.
37	C3	17	CONTROL	0	2	.	.	.
38	C6	1	CONTROL	0	2	.	.	.
39	C6	2	CONTROL	0	2	.	.	.
40	C6	3	CONTROL	0	2	.	.	.
41	C6	4	CONTROL	0	2	.	.	.
42	C6	5	CONTROL	0	2	.	.	.
43	C6	6	CONTROL	0	2	.	.	.
44	C6	7	CONTROL	0	2	.	.	.
45	C6	8	CONTROL	0	2	.	.	.
46	C6	9	CONTROL	0	2	.	.	.

47	C6	10	CONTROL	0	2	.	.	.
48	C6	11	CONTROL	0	2	.	.	.
49	C6	12	CONTROL	0	2	.	.	.
50	C6	13	CONTROL	0	2	.	.	.
51	C6	14	CONTROL	0	2	.	.	.
52	C6	15	CONTROL	0	2	.	.	.
53	C6	16	CONTROL	0	2	.	.	.
54	C6	17	CONTROL	0	2	.	.	.
55	C6	18	CONTROL	0	2	.	.	.
56	C6	19	CONTROL	0	2	.	.	.
57	C6	20	CONTROL	0	2	.	.	.
58	E1	1	ATRAZINE	0	2	17.134	.	.
59	E1	2	ATRAZINE	0	2	.	.	.
60	E1	3	ATRAZINE	0	2	.	.	.
61	E1	4	ATRAZINE	0	2	.	.	.
62	E1	5	ATRAZINE	0	2	.	.	.
63	E1	6	ATRAZINE	0	2	.	.	.
64	E1	7	ATRAZINE	0	2	.	.	.
65	E1	8	ATRAZINE	0	2	.	.	.
66	E3	1	ATRAZINE	0	2	217.908	.	.
67	E3	2	ATRAZINE	0	2	.	.	.
68	E3	3	ATRAZINE	0	2	1.978	.	.
69	E3	4	ATRAZINE	0	2	0.675	.	.
70	E3	5	ATRAZINE	0	2	9.357	9.5111	101.647

MEAN MALE AROMATASE ACTIVITY BY SITE AND ANIMAL NUMBER 153

Obs	Site	Ind_No	TREAT	_TYPE_	_FREQ_	A	STD	CV
71	E3	6	ATRAZINE	0	2	.	.	.
72	E3	7	ATRAZINE	0	2	2.707	.	.
73	E3	8	ATRAZINE	0	2	.	.	.
74	E3	9	ATRAZINE	0	2	6.979	.	.
75	E3	10	ATRAZINE	0	2	.	.	.
76	E4	1	ATRAZINE	0	2	19.360	7.9034	40.823
77	E4	2	ATRAZINE	0	2	12.020	2.7125	22.567
78	E4	3	ATRAZINE	0	2	4.930	1.5075	30.578
79	E4	4	ATRAZINE	0	2	11.255	1.5916	14.142
80	E4	5	ATRAZINE	0	2	6.312	1.5449	24.477
81	E4	6	ATRAZINE	0	2	.	.	.
82	E4	7	ATRAZINE	0	2	7.929	0.4772	6.018
83	E4	8	ATRAZINE	0	2	44.522	26.0913	58.603
84	E4	9	ATRAZINE	0	2	21.735	2.4152	11.112
85	E4	10	ATRAZINE	0	2	33.740	16.4240	48.678
86	E6	1	ATRAZINE	0	2	.	.	.
87	E6	2	ATRAZINE	0	2	.	.	.
88	E6	3	ATRAZINE	0	2	.	.	.
89	E6	4	ATRAZINE	0	2	.	.	.
90	E6	5	ATRAZINE	0	2	.	.	.
91	E6	6	ATRAZINE	0	2	.	.	.
92	E6	7	ATRAZINE	0	2	.	.	.
93	E6	8	ATRAZINE	0	2	.	.	.
94	E6	9	ATRAZINE	0	2	.	.	.
95	E6	10	ATRAZINE	0	2	.	.	.
96	E8	1	ATRAZINE	0	2	.	.	.
97	E8	2	ATRAZINE	0	2	.	.	.
98	E8	3	ATRAZINE	0	2	.	.	.
99	E8	4	ATRAZINE	0	2	.	.	.
100	E8	5	ATRAZINE	0	2	.	.	.
101	E8	6	ATRAZINE	0	2	.	.	.

ANOVA FOR MALE AROMATASE ACTIVITY BETWEEN SITES 154

The ANOVA Procedure

Class Level Information

Class	Levels	Values
Site	8	C1 C3 C6 E1 E3 E4 E6 E8

Number of observations 101

NOTE: Due to missing values, only 28 observations can be used in this analysis.

Dependent Variable: A

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	3	2160.42889	720.14296	0.42	0.7416
Error	24	41331.85569	1722.16065		
Corrected Total	27	43492.28458			

R-Square	Coeff Var	Root MSE	A Mean
0.049674	179.2665	41.49892	23.14929

Source	DF	Anova SS	Mean Square	F Value	Pr > F
Site	3	2160.428887	720.142962	0.42	0.7416

Levene's Test for Homogeneity of A Variance
ANOVA of Squared Deviations from Group Means

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Site	2	1.7871E8	89355288	2.78	0.0821
Error	24	7.717E8	32154336		

Bartlett's Test for Homogeneity of A Variance

Source	DF	Chi-Square	Pr > ChiSq
Site	2	34.2288	<.0001

NOTE: This test controls the Type I experimentwise error for comparisons of all treatments against a control.

Alpha	0.05
Error Degrees of Freedom	24
Error Mean Square	1722.161
Critical Value of Dunnett's t	2.54459

Comparisons significant at the 0.05 level are indicated by ***.

Site Comparison	Difference Between Means	Simultaneous 95% Confidence Limits	
E3 - C3	20.80	-32.00	73.60
E4 - C3	-1.16	-47.72	45.41
E1 - C3	-2.00	-111.91	107.91

The NPAR1WAY Procedure

Wilcoxon Scores (Rank Sums) for Variable A
Classified by Variable Site

Site	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
C3	12	199.0	174.00	21.540659	16.583333
E1	1	16.0	14.50	8.077747	16.000000
E3	6	54.0	87.00	17.860571	9.000000
E4	9	137.0	130.50	20.328551	15.222222

Kruskal-Wallis Test

Chi-Square	3.5546
DF	3
Pr > Chi-Square	0.3137

The NPAR1WAY Procedure

Median Scores (Number of Points Above Median) for Variable A
Classified by Variable Site

Site	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
C3	12	8.0	6.00	1.333333	0.666667
E1	1	1.0	0.50	0.500000	1.000000
E3	6	1.0	3.00	1.105542	0.166667
E4	9	4.0	4.50	1.258306	0.444444

Median One-Way Analysis

Chi-Square	4.9286
DF	3
Pr > Chi-Square	0.1771

MEAN MALE AROMATASE ACTIVITY BY TREATMENT 160

Obs	TREAT	_TYPE_	_FREQ_	A	STD	CV
1	ATRAZINE	0	88	22.6808	42.1873	186.004
2	CONTROL	0	114	20.3370	17.8629	87.834

ANOVA FOR MALE AROMATASE ACTIVITY ACROSS TREATMENTS 161

The ANOVA Procedure

Class Level Information

Class	Levels	Values
TREAT	2	ATRAZINE CONTROL

Number of observations 101

NOTE: Due to missing values, only 28 observations can be used in this analysis.

Dependent Variable: A

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	1	338.13513	338.13513	0.20	0.6555
Error	26	43154.14945	1659.77498		
Corrected Total	27	43492.28458			

R-Square	Coeff Var	Root MSE	A Mean
0.007775	175.9896	40.74034	23.14929

Source	DF	Anova SS	Mean Square	F Value	Pr > F
TREAT	1	338.1351305	338.1351305	0.20	0.6555

Levene's Test for Homogeneity of A Variance
ANOVA of Squared Deviations from Group Means

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
TREAT	1	40590974	40590974	0.85	0.3663
Error	26	1.2481E9	48003230		

Bartlett's Test for Homogeneity of A Variance

Source	DF	Chi-Square	Pr > ChiSq
TREAT	1	17.1290	<.0001

Dunnett's t Tests for A

NOTE: This test controls the Type I experimentwise error for comparisons of all treatments against a control.

Alpha	0.05
Error Degrees of Freedom	26
Error Mean Square	1659.775
Critical Value of Dunnett's t	2.05558

Comparisons significant at the 0.05 level are indicated by ***.

TREAT Comparison	Difference Between Means	Simultaneous 95% Confidence Limits	
CONTROL - ATRAZINE	-7.022	-39.003	24.958

Data Evaluation Report on Forms and Prevalence of Intersexuality and Effects of Environmental Contaminants on Sexuality in Cricket Frogs (*Acris crepitans*)

EPA MRID Number: None

Data Requirement:

EPA DP Barcode	None
EPA MRID	Not Assigned
EPA Guideline	Open Literature

Test material: **Purity:** not reported

Common name Atrazine
chemical name: IUPAC
CAS name 6-chloro-N-ethyl-N'-(1-methylethyl)-1,3,5-triazine-2,4-diamine
CAS No. 1912-24-9
synonyms
EPA PC Code: 80803

Thomas M. Steeger

Primary Reviewer: Thomas M. Steeger, Ph.D., Senior Biologist **Date:** May 1, 2003
Environmental Fate and Effects Division, ERB 4, U. S. Environmental Protection Agency

Joseph E. Tietge

Secondary Reviewer(s): Joseph E. Tietge, Research Aquatic Biologist **Date:** 5/1/03
Mid-Continent Ecology Division, National Health and Environmental Effects Research Laboratory (Duluth), U. S. Environmental Protection Agency

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Mary J. Frankenberry, Senior Statistician **Date:** 5/1/03
Environmental Fate and Effects Division, ERB 3, U. S. Environmental Protection Agency

EPA PC Code 080803

Date Evaluation Completed: 05/01/2003

CITATION: Reeder, A. L., G. L. Foley, D. K. Nichols, L. G. Hansen, B. Wikoff, S. Faeh, J. Eisold, M. B. Wheeler, R. Warner, J. E. Murphy, and V. R. Beasley 1998. Forms and prevalence of intersexuality and effects of environmental contaminants on sexuality in cricket frogs (*Acris crepitans*). Environmental Health Perspectives 106 (5):

EXECUTIVE SUMMARY:

In an effort to assess the prevalence of gonadal intersexuality in adult and juvenile cricket frogs *(Acris crepitans*) and to determine whether sexuality is altered in response to exposure to environmental contaminants, cricket frogs were collected over a three year period (1993 - 1995) in various locations throughout the state of Illinois. Additionally, water/sediment samples were collected at sampling sites in 1994 and 1995 to determine whether the prevalence of intersex could be related to chemical residues. In a separate study, cricket frogs were also collected at a site known to be contaminated with PCBs and PCDFs and the prevalence of intersex was determined relative to control sites.

Of the 55 adult and juvenile male and female frogs collected in 1993, 2 (3.6%) had both an ovary and testis. In the testis of one, spermatogenesis was normal; in the other, an immature ovary was present as well as a testis with no active spermatogenesis. Of the 243 frogs examined in 1994, 6 (2.5%) contained both ovary and testis; five of the affected animals had areas of normal spermatogenesis in the testis interspersed with oocytes. One animal had a mature ovary and mature testis with normal spermatogenesis. Of the 43 frogs examined in 1995, only one (2.3%) had an ovotestis. Across all three sampling years the prevalence of intersex was 2.8%. In specimens with an ovary on one side and an testis on the other, ovarian size ranged form well-developed mature female to extremely small with a few oocytes present.

Of the five sites where intersex was found, four had detectable atrazine. Of the four sites where no intersex was observed, only one contained detectable levels of atrazine. According to the authors the relationship between detection of atrazine and prevalence of intersex "approached significance" ($P = 0.07$). At one site treated with copper sulfate in 1994, 1 frog of 33 collected had an ovotestis. In 1995, no relationship between the detection of atrazine and the prevalence of intersex. No intersex was identified in frogs collected from a pond treated with endothall in 1995. Lead residues measured in 1994 and 1995 were not associated with the prevalence of intersex.

Of the frogs collected from PCB and control sites, only 1 frog with an ovotestis was identified from the control. Sex ratios differed significantly (probability not given) between contaminated and control sites. In 13 juveniles from control and 13 from contaminated sites, gonadal tissue was immature and could not be identified for histological preparation. According to the authors, the association between sex ratios of PCB/PCDF contaminated and control groups revealed a significant difference ($p = 0.0007$).

While a wide range of chemical residue analyses were conducted, no data are provided on the results of these analyses. It is not clear why the study focuses primarily on atrazine; however, the authors suggest that there may be a trend between atrazine residues and the proportion of animals exhibiting intersex. The only statistically significant relationship though was between sex ratios in PCB/PCDF contaminated sites relative to controls; however, the sample size for making this determination was low (n=4).

In this paper, Reeder *et al.* (1998) discussed the range of chemical residues in the field collection sites and how these chemicals, combined with environmental conditions, could impact gonadal development. These factors contributed to the limited utility of this study because the investigation did not demonstrate a significant effect of chemical residues on the prevalence of intersex in cricket frogs. This study underscored the need to have focused study designs with sufficient power in terms of sample size to discriminate effects if they exist. Also, the report acknowledged that little is known about natural intersex rates in cricket frogs. Without a better understanding of the biology of the cricket frog and the toxicological phenomenon being examined, it is difficult to interpret the significance of the reported observations.

I. MATERIALS AND METHODS

GUIDELINE FOLLOWED: Nonguideline Study
COMPLIANCE: Not conducted under full Good Laboratory Practices

A. MATERIALS:

1. Test Material Atrazine

Description:

Lot No./Batch No. : Not reported

Purity:
Stability of Compound Under Test Conditions: Not reported

Storage conditions of test chemicals:_ Not reported

2. Test organism:

Species: Cricket frogs (Acris crepitans)

Age at test initiation:
Weight at study initiation: (mean and range) not reported
Length at study initiation: (mean and range) not reported

Source: Field-collected along pond banks in sites distributed across Illinois

B. STUDY DESIGN:

Objective: to assess the prevalence of gonadal intersexuality in adult and juvenile cricket frogs *(Acris crepitans*) and to determine whether sexuality is altered in response to exposure to environmental contaminants

1. Experimental Conditions

a) **Range-finding Study**: In 1993, a pilot study focused on pond site identification and preliminary assessment of histologic lesions in Illinois cricket frogs, 20 sites selected.

b) Definitive Study

In 1994 and 1995 frogs were collected from specific sites based on suitable habitat and presence of cricket frogs; ponds were of comparable size and were distributed across the state of Illinois.

Table 1 . Experimental Parameters

Parameter	Details
Acclimation: period: conditions: (same as test or not) Feeding: Health: (any mortality observed)	NA
Duration of the test	NA
Test condition static/flow through Type of dilution system- for flow through method. Renewal rate for static renewal	NA
Aeration, if any	NA
<u>Test vessel</u> Material: (glass/stainless steel) Size: Fill volume:	NA
Source of dilution water Quality:	NA

Parameter	Details
Water parameters: Hardness pH Dissolved oxygen Total Organic carbon Particulate Matter Ammonia Nitrite Metals Pesticides Chlorine Temperature Salinity Intervals of water quality measurement	NA
Number of replicates/groups: negative control: 0.004% ethanol treated ones:	NA
Number of organisms per replicate /groups:	In 1993, gonads of 50 intact cricket frogs from 18 sites. In 1994, gonads of 242 frogs examined from 8 sites (24 - 39 frogs from the various sites). In 1995, gonads of 40 frogs examined from 7 sites (4 - 7 frogs per site).
Biomass loading rate	NA
Test concentrations: nominal:	NA
Solvent (type, percentage, if used)	NA
Lighting	NA
Feeding	NA
Recovery of chemical Level of Quantitation Level of Detection	NA

Parameter	Details
Positive control {if used, indicate the chemical and concentrations}	NA
Other parameters, if any	NA

2. Observations:

Table 2: Observations

Criteria	Details
Parameters measured including the sublethal effects/toxicity symptoms	prevalence of gonadal abnormalities in terms of testicular oocytes.
Observation intervals	NA
Were raw data included?	No
Other observations, if any	

Study A

In 1994, eight sites with similar size studied (5 in south, 2 in central and 1 in northeast Illinois). In 1995, seven of the ponds studied in 1994 and one pond in the agricultural area of east-central Illinois, studied.

In 1993, gonads of 50 intact cricket frogs from 18 sites in east-central and southern Illinois examined for gross morphology. In 1994, gonads of 242 frogs examined from 8 sites (24 - 39 frogs from the various sites). In 1995, gonads of 40 frogs examined from 7 sites (4 - 7 frogs per site).

Animals from 1993 subject to whole body histologic sections while samples from 1994 and 1995 were subject to gonad histology.

Composite water and sediment samples collected in acetone-rinsed containers from multiple locations in each pond studied in 1994 and 1995. Samples collected at the end of May and again in late July/early August in 1994 and at the end of May in 1995. Additional water samples were collected at sites 11 and 12 in June 1998 following an aquatic plant kill. Water analyzed (State of Illinois Animal Disease Laboratory, Centralia, IL) for herbicides, fungicides, insecticides, PCBs and metals (**Table 1; source: Reeder *et al.* 1998**).

<u>Study B</u>

In mid-July 1995, 16 juvenile cricket frogs collected from three sites in Crab Orchard National Wildlife Refuge in southern Illinois where point contamination with PCBs, PCDFs, and PCDDs had previously occurred. On the same sampling days, 16 juvenile frogs also collected from each of three control sites located outside the area of PCB and PCDF contamination but within a 16-km radius of the Crab Orchard site.

Fisher's Exact Test used to compare prevalence of intersex gonads and detection of atrazine at those sites. Fisher's Exact test also used to compare sex ratios among control and PCB/PCDF sites using SigmaStat software.

Table 1. Compounds analyzed in water and sediment samples (water detection limits in parentheses, µg/l)

Herbicides	Insecticides
Alachlor (5)	Carbamates
Ametryn (1)	Aldicarb (10)
Atrazine (0.5)	Aminocarb (5)
Barban (5)	Bendiocarb (5)
Bifenox (5)	Carbaryl (5)
Bromacil (1)	Carbofuran (5)
Butachlor (5)	Lannate (5)
Butylate (5)	Methiocarb (5)
Chloropropham (5)	Oxamyl (5)
Chlorthal (5)	Organochlorine
Cyanazine (0.5)	Aldrin (0.5)
Diclofop (5)	Chlordane (0.5)
Dinitramine (5)	DDD (0.5)
Dipropetryn (5)	DDE (0.5)
Diuron (5)	DDT (0.5)
EPTC (5)	Dieldrin (0.5)
Fluchloralin (5)	Endosulfan (0.5)
Hexazinone (5)	Endrin (0.5)
Linuron (5)	Heptachlor (0.5)
Metolachlor (5)	Heptachlor epoxide (0.5)
Metribuzin (5)	Lindane and isomers (0.5)
Monuron (5)	Methoxychlor (0.5)
Napropamide (5)	Mirex (0.5)
Naptalam (5)	PCBs (0.5)
Oryzalin (5)	Organophosphorous
Pebulate (5)	Chlorpyrifos (1)
Pendimethalin (5)	Diazinon (1)
Profluralin (5)	Dimethoate (1)
Prometon (5)	Disulfoton (1)
Prometryn (5)	Ethoprop (1)
Propanil (5)	Ethyl parathion (1)
Propazine (5)	Fenchlorphos (1)
Propham (5)	Fenthion (1)
Simazine (1)	Fonofos (1)
Terbuthylazine (5)	Isofenphos (1)
Terbutryn (5)	Malathion (1)
Trifluralin (5)	Methidathion (1)
	Methyl parathion (1)
	Mevinphos (1)
	Phorate (1)
	Terbufos (1)
	Trichlorfon (1)
	Fungicides
	Benomyl (5)
	Hexachlorobenzene (0.5)
	Thiobencarb (10)
	Metals
	Lead (5, 0.1 mg/kg[a])
	Mercury (0.1 µg/kg[a])

[a]Sediment detection limits.

II. <u>RESULTS</u> and <u>DISCUSSION</u>:

<u>Study A</u>

Although cricket frog testicular tissue is typically heavily pigmented, about 20% of males had testes with reduced or absent pigmentation particularly on the right side; however, normal spermatogenesis was observed in both pigmented and nonpigmented testes. Mature females contained large egg mass with bipolar eggs (tan/black coloring).

Of the 55 adult and juvenile male and female frogs collected in 1993, 2 (3.6%) had both an ovary and testis. In the testis of one, spermatogenesis was normal; in the other, an immature ovary was present as well as a testis with no active spermatogenesis. Of the 243 frogs examined in 1994, 6 (2.5%) contained both ovary and testis; five of the affected animals had areas of normal spermatogenesis in the testis interspersed with oocytes. One animal had a mature ovary and mature testis with normal spermatogenesis. Of the 43 frogs examined in 1995, only one (2.3%) had an ovotestis. Across all three sampling years, the prevalence of intersex was 2.8% (report claims 2.6%). In specimens with an ovary on one side and an testis on the other, ovarian size ranged form well-developed mature female to extremely small with a few oocytes present.

Of the five sites where intersex was found, four had detectable atrazine. Of the four sites where no intersex was observed, only one contained detectable levels of atrazine.

According to the authors the relationship between detection of atrazine and prevalence of intersex "approached significance" (P = 0.07). At one site treated with copper sulfate in 1994, 1 frog of 33 collected had an ovotestis. In 1995, no relationship between the detection of atrazine and the prevalence of intersex. No intersex was identified in frogs collected from a pond treated with endothall in 1995. Concentrations of lead from both years not associated with intersex either.

Study B

Of the frogs collected from PCB and control sites, only 1 frog with an ovotestis was identified from the control. Sex ratios differed significantly (probability not given) between contaminated and control sites. In 13 juveniles from control and 13 from contaminated sites, gonadal tissue was immature and could not be identified for histological preparation. According to the authors, the association between sex ratios of PCB/PCDF contaminated and control groups revealed a significant difference (p = 0.0007).

F. REVIEWER'S COMMENTS:

It is not clear what was driving the sampling design used in this study. Sample size varied considerably between years: 1993 (55 frogs), 1994 (243 frogs) and 1995 (43 frogs).

There are several discrepancies between the tables and the text. In discussing the prevalence of intersex relative to atrazine, the authors refer to 5 sites with residues and 4 sites without residues in the 1994 sampling; however, according to the methods section only 8 sites (not 9) were sampled in 1994.

In study A, a wide range of chemicals were analyzed in both water and sediment at each of the collection sites in 1994 and 1995; however, the results section does not discuss the outcome of these analyses but rather focuses primarily on atrazine thus implying that it was either the only chemical were residues were detected or that it was the only chemical residues where the researchers could identify a significant relationship. Since no data are provided on residue levels, it isn't possible for the Agency to verify whether a correlation existed between atrazine residues and the prevalence of intersex; however, the results section suggests that if a relationship did exist in 1994, it was transient since no relationship existed in 1995. Further, it isn't clear whether the lack of a "relationship" between atrazine residues in 1995 and atrazine residues was a result of only one intersex animal being found in spite of atrazine residues or whether there were no detectable atrazine residues. The article implies that the authors attempted to correlate atrazine residues only to cases where intersex was determined. If this is the case then the analysis is somewhat biased since zero prevalence would not be captured. Atrazine residues at each sites should have been compared to the rate of intersex at each site; however, it doesn't appear that the study was designed to anticipate such an analysis. The utility of calculating an overall prevalence of abnormalities seems unsound, as it unnecessarily combines data from sites identified as contaminated or control.

In study B, the methods section suggests that they collected 16 frogs from control and from contaminated sites. The results section states that the gonads of 13 frogs from each location were too immature to determine sex. This implies that over 80% of the animals were too immature to determine sex. Therefore, the effective sample size for determining sex ratio differences was low with three animals in each group.

Presentation of the analytical chemistry data is not clear...are blank entries indicative of no analyses or no measurable quantities?. The authors note the variety of chemical residues measured in the field studies and how varying levels of response to the wide range of chemicals might influence the frog's sensitivity to the chemicals. Classifying a site categorically as atrazine contaminated based on the sampling plan is misleading. The real question is: what were the atrazine concentrations during the developmental stage of interest? Given this lack of information, it is difficult to accept the correlational association of gonadal abnormalities and atrazine presence, as it has no basis in the biology nor toxicology. Additionally the authors note how environmental factors such as temperature may impact gonadal development. Given the number of potentially confounding effects, it is difficult to place much weight on the statistical relationships developed in what amounts to a reconnaissance survey. The factors contribute to the limited utility of this study since the study does not demonstrate a significant affect of chemical residues on the prevalence of intersex in cricket frogs. The study underscores to need to have focused study designs with sufficient power in terms of sample size to discriminate effects should they exist.

The report admits in the introduction that virtually nothing is known about intersex conditions in cricket frogs. Perhaps cricket frogs exhibit ovotestes normally at some low level and the presence and absence of this condition at any one site is a function of small sample sizes. In general, this study is another one that puts the "cart ahead of the horse." That is, they conducted a field study, replete with complications and uncertainties without having a firm understanding of either the biology of the organism that they were studying or the toxicological phenomenon that they attempted to study. It clearly does not contribute to the understanding of whether or not atrazine poses any reproductive risk to anurans.

Data Evaluation Report on Hermaphroditic, Demasculinized Frogs After Exposure to the Herbicide Atrazine at Low Ecologically Relevant Doses

EPA MRID Number: None

Data Requirement::

EPA DP Barcode None

EPA MRID Not Assigned
EPA Guideline Open Literature

Test material: **Purity:** not reported

Common name Atrazine
Chemical name: IUPAC
CAS name 6-chloro-N-ethyl-N'-(1-methylethyl)-1,3,5-triazine-2,4-diamine
CAS No. 1912-24-9
Synonyms
EPA PC Code: 80803

Thomas M Steeger

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U. S. Environmental Protection Agency

Joseph E Tietge

Secondary Reviewer(s): Joseph E. Tietge, M.S., Research Aquatic Biologist **Date:** 5/1/03
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Stephanie Irene, Ph.D., Senior Advisor **Date:** 5/6/03
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Mary Frankenberry

Mary Frankenberry, Senior Statistician **Date:** 5/5/03
Environmental Fate and Effects Division, ERB 3, U. S. Environmental Protection Agency

EPA PC Code 080803

TMS 6/2/03

Date Evaluation Completed: ~~05~~/30/2003

CITATION: Hayes, T.B., A. Collins, M. Lee, M. Mendoza, N. Noriega, A. A. Stuart, A. Vonk. 2002. Hermaphroditic, demasculinized frogs after exposure to the herbicide atrazine at low ecologically relevant doses. *Proceedings of the National Academy of Science*, 99 (8): 5476 - 5480.

EXECUTIVE SUMMARY:

The objective of this study was to test the hypothesis that atrazine, at ecologically relevant low doses, may interfere with metamorphosis and sex differentiation via endocrine-disrupting mechanisms. African clawed frogs (*Xenopus laevis*) were exposed to atrazine concentrations ranging from 0.01 to 200 µg/L from 96-hr post-hatch through complete tail resorption (Nieuwkoop-Faber Stage 66). At the end of the exposure period, animal growth (length and weight), time to metamorphosis, gonadal abnormalities and size (cross-sectional diameter) of the larynx dilator muscle were recorded. Exposure to atrazine concentrations 0.1 µg/L resulted in gonadal abnormalities in 16 - 20% of the animals at all doses tested except for 0.1 ug/L. Abnormalities included multiple gonads or hermaphrodites (multiple testes and ovaries in the same animal). These abnormalities were not observed in the controls. In general, males typically exhibited larger diameter laryngeal muscle diameters than females. In this study, though, atrazine at concentrations as low as 1 µg/L (1 ppb), significantly decrease the proportion of males that were at or above the mean laryngeal size in the control males. According to the authors, these results suggested a threshold effect at 1 µg/L in which 80% of the exposed males exhibited larynx sizes below the average. The authors hypothesized that the co-occurrence of oocytes and testicular tissue (hermaphroditism) and the decreased male larynx muscle size (demasculinizing) were consistent with increased endogenous estrogen concentrations and that one possible mechanism would be through increased aromatase activity. In support of their hypothesis, the researchers demonstrated that adult male *Xenopus* exposed to atrazine at 25 µg/L showed significantly reduced plasma testosterone.

This study states that the results have been repeatedly verified, but additional data have not yet been provided in the open literature or submitted to EPA. It would be helpful if supporting studies were available to verify the results of this specific study. In addition, more information on the measurement endpoints, effects in the field, percentage of males and females in the study, numbers of frogs exhibiting abnormalities, and sensitivity of *Xenopus laevis* compared to North American frogs would be useful. Also, the lack of a dose- response relative to the phenomenon of hermaphroditism makes it difficult to interpret cause-effect relationships. Although there appears to be a dose-dependent reduction in laryngeal muscle area relative to atrazine concentrations, the reliance on the proportion of animals falling below the average is an indirect measure of the effect. A direct comparison of measured laryngeal muscle area between controls and exposed animals would further clarify the magnitude and extent of any developmental effects. Information on how diminished dilator muscle area or the gonadal deformities might relate to the reproductive success, growth or survival of the affected species in the environment would also provide further insights on the ecological relevancy of effects.
This laboratory study was not conducted for the purposes of reregistration and many of the details typically monitored in a study conducted under Good Laboratory Practice standards were not recorded.

I. MATERIALS AND METHODS

GUIDELINE FOLLOWED: Nonguideline study
COMPLIANCE: Not conducted under full Good Laboratory Practices

A. MATERIALS:

1. Test Material Atrazine

Description: Not reported

Lot No./Batch No. : Not reported

Purity: Not reported
Stability of compound under test conditions: Not reported

Storage conditions of test chemicals: Not reported

2. Test organism:

Species: African clawed frog (*Xenopus laevis*)

Age at test initiation Experiment 1 and 2: 96-hr larvae; Experiment 3: adults (age not reported)
Weight at study initiation: (mean and range) not reported
Length at study initiation: (mean and range) not reported

Source: Eggs and sperm obtained from three adults (3 males and 3 females) from long-term captive colony maintained at the University of California, Berkeley, for Experiment 1 and from adults obtained from Nasco (Fort Atkinson, WI) for Experiment 2. Adults from long-term captive colony maintained at UC Berkeley for Experiment 3.

B. STUDY DESIGN:

Objective: To test the hypothesis that atrazine may interfere with metamorphosis and sex differentiation at ecologically relevant low doses via endocrine-disrupting mechanisms.

1. Experimental Conditions

a) **Range-finding Study**: Not reported
b) **Definitive Study**

Table 1 . Experimental Parameters

Parameter	Details
Acclimation: period: Conditions: (same as test or not) Feeding: Health: (any mortality observed)	Experiments 1 and 2: Embryos hatched in 0.3x modified mammalian Ringer's solution; at 96-hrs post-hatch, larvae transferred to aerated 10% Holtfreter's solution. Fed solution of ground Purina rabbit chow daily. No mortality reported. Experiment 3: Adults acclimated in 10% Holtfreter's solution for 5 days, unaerated; animals fed Purina trout chow daily; water renewed every 72 hours.
Duration of the test	Experiment 1 and 2: Hatching (NF Stage 48) until complete tail resorption (NF Sate 66) Experiment 3: Adults (age not reported) exposed for 46 days.
Test condition static/flow-through Type of dilution system- for flow-through method. Renewal rate for static renewal	 static renewal NA complete exposure solution change every 72 hours
Aeration, if any	Experiments 1 and 2 with larvae employed aeration; Experiment 3 with adults did not employ aeration
Test vessel Material: (glass/stainless steel) Size: Fill volume:	 plastic cages (personal communication, T. Hayes, 2002) 4 L
Source of dilution water quality:	Deionized, distilled water (personal communication, T. Hayes, 2002)

Parameter	Details
<u>Water parameters</u>: Hardness pH Dissolved oxygen Total Organic carbon Particulate Matter Ammonia Nitrite Metals Pesticides Chlorine Temperature Salinity Intervals of water quality measurement	 not reported not reported not reported not reported not reported not reported not reported not reported not reported not reported 22°C not reported not reported
Number of replicates/groups: negative control: 0.004% ethanol treated ones:	<u>Experiment 1 and 2</u>: 3 replicates
Number of organisms per replicate /group:	<u>Experiment 1 and 2:</u> 30 larvae per replicate
Biomass loading rate	<u>Experiment 1 and 2:</u> 30 larvae/4 L
Test concentrations: nominal:	 <u>Experiment 1:</u> 0.01, 0.1, 1.0, 10.0, and 25 µg/L <u>Experiment 2:</u> 0.1, 0.4, 0.8, 1.0, 25, and 200 µg/L <u>Experiment 3:</u> 25 µg/L
Solvent (type, percentage, if used)	<u>Experiments 1 and 2:</u> 0.004% ethanol in 10% Holtfreter's solution. <u>Experiment 3:</u> 10% Holtfreter's solution Holtfreter's medium:
Lighting	12 hrs light, 12 hrs dark
Feeding	<u>Experiments 1 and 2:</u> Purina rabbit chow *ad libitum* (personal communication, T. Hayes, 2002) <u>Experiment 3:</u> Purina trout chow daily

Parameter	Details
Recovery of chemical Level of Quantitation Level of Detection	Concentrations confirmed by PTRL West, Richmond, Ca and Iowa Hygenic Laboratory, Univ. of Iowa, Iowa City, IO).. Not reported. Not reported.
Positive control {if used, indicate the chemical and concentrations}	not reported?
Other parameters, if any	

2. Observations:

Table 2: Observations

Criteria	**Details**
Parameters measured including the sublethal effects/toxicity symptoms	Experiment 1 and 2: mortality, time to metamorphosis, weight and length at metamorphosis, sex based on gross morphology (all animals) and histology (10 animals/tank), laryngeal size based on largest cross-sectional area of *M. dilator larngis* (transverse histology from 10 males and 10 females from each replicate).
Observation intervals	Not reported
Were raw data included?	No
Other observations, if any	Experiment 3: plasma testosterone in blood collected by decapitation using radioimmuno-assay. Testosterone antisera obtained from Endocrine Sciences (Calabasas, CA).

II. RESULTS and DISCUSSION:

Statistical analysis was conducted using SYSTAT® (SPSS, Chicago); sex ratios and mortality were determined using a G test with Wilkin's g-adjustment ; and time to metamorphosis and growth (length and weight at metamorphosis) were analyzed using ANOVA. Animals were scored based on the size of their larynx in relationship to the mean laryngeal size for controls. After scoring, a G test was conducted to determine the affect of atrazine treatment followed by Kendall's ranked coefficient to determine whether the percentage of below-average animals varied with the dose of atrazine.

Experiments 1 and 2:

At the doses tested, atrazine exposure had no significant ($P > 0.05$) effect on mortality, time to metamorphosis, length, or weight at metamorphosis. Males and females were sexually differentiated at metamorphosis based on gonadal morphology and histology. All atrazine doses tested except 0.01 µg/L produced gonadal abnormalities in 16 - 20% of the animals. Abnormalities included multiple gonads or hermaphrodites (multiple testes and ovaries. These abnormalities were not observed in the controls. The paper contains histological sections through the gonads of an animal exposed to 1 µg/L atrazine that was classified as a hermaphrodite. The sections in the histological slides showed a transition from bilateral testicular tissue to ovarian tissue, moving caudally along the gonads.

Control males had larger larynges than females at metamorphosis, but males exposed to 1 µg/L had reduced larynges in both Experiments 1 and 2. In the caption of Figure 3, the authors noted that atrazine 1 µg/L reduced laryngeal size in males but did not affect females. Larynges of animals in Experiment 2 were larger than in Experiment 1, suggesting a population difference in absolute size of the larynges. Similar to Experiment 1 though, exposure to atrazine at 1 µg/L significantly reduced laryngeal size in males. Log-transformed percent of animals above control mean laryngeal size indicated that atrazine exposure 1 µg/L significantly decreased the proportion of males that were at or above the mean control males (G test, $p < 0.05$). It also suggested a threshold effect at 1 µg/L in which 80% of the exposed males were below average. The percentage of below-average animals suggested a dose effect with increasing atrazine doses (Kendal rank coefficient; $p < 0.01$).

<u>Experiment 3:</u>

An extrapolatation of Figure 4 of the paper indicated that plasma testosterone levels in control males averaged 4 ng/mL. Plasma testosterone in atrazine-treated males and control females averaged roughly 0.1 and 0.2 ng/mL, respectively, and were significantly lower (ANOVA, $P < 0.05$) than control males.

The authors hypothesized that the 16 - 20% incidence of multiple gonads/hermaphroditism and smaller than average larynxes in males were caused by atrazine- disrupting steroidogenesis. They also concluded that sexually mature males suffered a 10-fold decrease in plasma testosterone. According to the authors, the current findings suggested that atrazine inhibits testosterone and induces estrogen synthesis by an induction of aromatase and consequent transformation of androgens (testosterone) to estrogen. They hypothesized that the loss of masculine features, *i.e.*, decreased laryngeal muscle size, may be due to decreased androgens, and the induction of ovaries may result from increased estrogen synthesis and secretion. According to the authors, the current data raised concerns regarding the effects of atrazine on amphibians: exposure to 0.1 µg/L produced hermaphrodites and exposure to 1 µg/L resulted in a reduction in laryngeal size.

F. <u>REVIEWER'S COMMENTS</u>:

This nonguideline, published study was useful in identifying a potential hazard to amphibians and presented information on measurement endpoints, such as gonadal deformities and laryngeal muscle diameter. The study, however, did not show a clear dose response that demonstrates a causal relationship between atrazine exposure and developmental effects. While the paper states that the study results have been repeated in 51 trials, the Agency did not receive these replicated data. Results of these repeated trials would help the Agency verify the results found in this study.

Because of the variability and nature of the measurement endpoints, the statistical analyses in this study relied heavily on nonparametric methods. Access to the raw data supporting this study would help the Agency to verify the study's conclusions regarding mortality, time to metamorphosis, growth, gonadal development, laryngeal diameter (size) or plasma testosterone levels. Although the range of gonadal abnormalities was provided, the prevalence of gonadal effects is difficult to interpret. EPA needs more specific data on the range of gonadal abnormalities, the incidence for each dose tested, the relative percentages of males and females, the numbers of animals involved in the 16-20% abnormality incidence, and the impact of gonadal abnormalities on reproductive success in the environment. Without this information, it is difficult to interpret the error bars in Figure 4 of the paper and to establish the ecological relevancy of the data. EPA also needs clarifying information concerning the sample sizes involved in the analyses, the number of doses samples and the method of blood collection (i.e., decapitation could contaminate blood samples).

In its evaluation of this study, the Agency had questions concerning the use of ethanol as a co-solvent. Since atrazine concentrations used in these studies were less than the 30 mg/L water solubility limit, a co-solvent should not be necessary. Previous studies conducted by the authors (personal communication: Tyrone Hayes 2002) used dihydrotestosterone and 17-β estradiol as positive controls and required ethanol as a co-solvent. The current study, though, did not utilize these steroids and a co-solvent should not have been necessary. The Agency also has questions concerning the conditions of the study. The study states that 30 organisms with an estimated maximal weight of 1.5 g were held in 4 liters of test solution. These conditions would result in a loading rate of 11.3 g/L, which is 27 times the recommended loading rate for static renewal toxicity tests.

While the paper discusses that the study results have been repeated in 51 trials, none of the data are presented; therefore, it is not possible to gauge the researchers' success at replicating the current results.

Although only the range of gonadal abnormalities (16 - 20%) is provided, no information is presented on what the incidence was for each dose tested. The data suggest that there was a threshold effect level at 0.1 μg/L but that the effect did not increase with increasing dose.

While the authors suggest that up-regulation of aromatase activity is a plausible rationale for the observed effects and that reduced testosterone levels in atrazine treated adult male *Xenopus* further support this hypothesis, no direct measure of aromatase activity. Further testing with additional dose concentrations is a needed to establish a causal relationship between atrazine exposure and gonadal effects in amphibians. is provided nor can a dose response be established. Additionally, if aromatase activity were increased and there was subsequent decline in testosterone, estrogen levels would likely increase; however, there are no data available on the estrogen concentrations. Since a single concentration (25 μg/L) of atrazine was used to test the hypothesis concerning aromatase, it isn't possible to establish a dose response.

The ecological relevancy of this data is not clear. No information is available on whether similar gonadal abnormalities impact reproductive success in the environment.

The lab study utilizes relatively sub-standard conditions in that 30 organisms with an estimated maximal weight of 1.5 g were held in 4 liters of test solution. This results in a loading rate of 11.3 g/L, which is 27 times the recommended loading rate for static renewal toxicity tests.

As reported, the prevalence of gonadal effects is difficult to interpret. The results section apparently combines the results of both larval studies and fails to provide dose response information and any indication of variance. Since the presentation of dose response data is a fundamental standard for toxicological studies, this omission is curious, and suggests that the report is incomplete for some reason. Also, what is the relative contribution of the two major abnormality types to the overall prevalence of gonadal abnormalities? There are no data presented on the relative percentages of males and females in the study, nor any other data indicating numbers on which the apparent 16-20% abnormality incidence was calculated.

Results from the adult atrazine exposure do not indicate the sample sizes involved in the analysis. In fact, it does not indicate the basic information on how many males were exposed or held as controls. Since this information is not available, one cannot make sense of the error bars (what are they representing?) in Figure 4, nor whether or not this is a credible analysis. In the RIA section of the Methods & Materials, the report states that plasma was sampled at 3 doses. However, the Adult Treatments section indicates that only one dose was utilized. The method of blood collection, decapitation, seems rather crude and likely to contaminate blood with other bodily fluids, which could confound the analysis. A more acceptable approach, for example, would be a cardiac puncture.

H. REFERENCES:

Nieuwkoop, P. D. and J. Faber. 1994. Normal table of *Xenopus laevis* (Daudin). North-Holland Publishing Company, Amsterdam.

Data Evaluation Report on Atrazine-Induced Hermaphroditism at 0.1 ppb in American Leopard Frogs (*Rana pipiens*): Laboratory and Field Evidence

EPA MRID Number: None

Data Requirement:

EPA DP Barcode	None
EPA MRID	Not Assigned
EPA Guideline	Open Literature

Test material: **Purity:** 98%

Common name Atrazine
Chemical name: IUPAC
CAS name 6-chloro-N-ethyl-N'-(1-methylethyl)-1,3,5-triazine-2,4-diamine
CAS No. 1912-24-9
Synonyms
EPA PC Code: 80803

Thomas M Steeger

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Environmental Fate and Effects Division, ERB 4, U. S. Environmental Protection Agency

Joseph E. Tietge

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EPA PC Code 080803

TMS 6/2/03
Date Evaluation Completed: 04/29/2003

CITATION: Hayes, T. B., K. Haston, M. Tsui, A. Hoang, C. Haeffele and A. Vonk. 2002. Atrazine-induced hermaphroditism at 0.1 ppb in American leopard frogs (*Rana pipiens*): laboratory and field evidence. Environmental Health Perspectives

EXECUTIVE SUMMARY:

The objective of this study was to examine the effects of atrazine on leopard frogs (*Rana pipiens*) under controlled laboratory conditions, and once the effects were identified to examine wild *R. pipiens* from a variety of habitats in areas with reportedly low atrazine use and high atrazine use. Water samples were also collected at each field site to determine atrazine exposure. The combination of both laboratory and field studies was intended to address the ecological significance and relevance of the initial laboratory studies.

In the laboratory, leopard frog larvae were exposed from 48-hrs post-hatch through complete tail resorption (NF-Stage 66) to atrazine at 0.1 and 25 µg/L in 0.0036% ethanol in 10% Holtfreter's solution. Animals were sacrificed; gross morphology and histological analysis of gonads revealed that 36% and 12% of the males treated with atrazine at 0.1 and 25 µg/L, respectively, suffered form gonadal dysgenesis (under-developed testes with poorly structured, closed lobules and low to absent germ cells). Further, 29% of the 0.1 µg/L and 8% of the 25 µg/L animals displayed varying degrees of sex reversal; testicular lobules of sex-reversed males contained oocytes, and males that metamorphosed later contained large numbers of oocytes. In a few cases, testicular oocytes were reported to be vitellogenic, *i.e.*, contained yolk.

In a field reconnaissance survey of leopard frogs in four low atrazine-use and four high atrazine-use sites, testicular oocytes were identified in males from seven of the eight collection sites. All sites with atrazine levels exceeding 0.2 µg/L had males that displayed sex-reversal similar to those abnormalities induced by atrazine in the lab. The highest incidence (92%) and most advanced cases of hermaphroditism were observed in animals collected from the North Platte River in Wyoming where atrazine residues found in water samples were among the lowest recorded. At sites with similar atrazine residues, the incidence of gonadal abnormalities varied considerably suggesting that there was not a clear pattern of response. Even at sites where no residues were reported, the incidence of testicular oogenesis appeared to be as high as 18%. Additionally, atrazine residues reported for each of the sampling sites may not be reflective of actual exposure conditions during larval development. Although young frogs were reportedly sampled, there is no information to support when the animals may have undergone metamorphosis relative to the atrazine residue analysis.

Although the researchers conducted pesticide residue analysis for chemicals that were reportedly used in the watershed, the scope of chemical analyses was on a site-by-site basis. As the authors noted, even atrazine was present where it was not used, and it is likely that other chemical contaminants may have been present. Chemical profiles of the sites and information on the morphoedapic characteristics of the sites would have shed light on the comparability of the study sites.

In their paper, the study authors suggested that the enhanced response at lower doses was consistent with low-dose effects reported for other endocrine-disrupting chemicals; however, the data did not show a clear dose response pattern. In a previous study using *Xenopus* (Hayes *et al.* 2002), there appeared to be a threshold effect for testicular abnormalities at 0.1 µg/L but the response appeared to remain steady across increasing concentrations of atrazine with 16 - 20% of the males exhibiting gonadal abnormalities. In the laboratory study, only two doses of atrazine were tested, and the field study did not indicate a clear trend. From these two studies, the study authors concluded there is an "inverted-U" (parabolic) dose response curve.

I. MATERIALS AND METHODS

GUIDELINE FOLLOWED: Nonguideline Study
COMPLIANCE: Not conducted under full Good Laboratory Practices

A. MATERIALS:

1. Test Material Atrazine

Description: Chemservice, Chester, PA

Lot No./Batch No. : Not reported

Purity: 98%
Stability of compound under test conditions: Not reported

Storage conditions of test chemicals: Not reported

2. Test organism:

Species: American leopard frog *(Rana pipiens)*

Age at test initiation: 48-hrs post-hatch
Weight at study initiation: (mean and range) not reported
Length at study initiation: (mean and range) not reported

Source: Leopard frogs [eggs] obtained from Sensiba Marsh, Brown County, Wisconsin.

B. STUDY DESIGN:

Objective: To examine the effects of atrazine on leopard frogs (*Rana pipiens*) under controlled laboratory conditions and once the effects were identified to examine wild *Rana pipiens* from a variety of habitats in areas with reportedly low atrazine use and high atrazine use. Water samples were also collected at each field site to determine level of atrazine. The combination of both laboratory and field studies was intended to address the ecological significance and relevance of the initial laboratory studies.

1. Experimental Conditions

a) **Range-finding Study**: Not reported
b) **Definitive Study**

Table 1 . Experimental Parameters

Parameter	Details
Acclimation: period: Conditions: (same as test or not) Feeding: Health: (any mortality observed)	Leopard frog eggs were allowed to hatch (conditions not stated) and then apportioned to rearing tanks.
Duration of the test	48-hrs post-hatch through Nieuwkoop-Faber (NF)Stage 66
Test condition static/flow- through Type of dilution system for flow-through method. Renewal rate for static renewal	 static renewal NA complete exposure solution change every 72 hours
Aeration, if any	exposure tanks aerated
<u>Test vessel</u> Material: (glass/stainless steel) Size: Fill volume:	 plastic cages (personal communication, T. Hayes, 2002) 4 L
Source of dilution water quality:	Deionized, distilled water (personal communication, T. Hayes, 2002)

Parameter	Details
Water parameters: Hardness pH Dissolved oxygen Total Organic carbon Particulate Matter Ammonia Nitrite Metals Pesticides Chlorine Temperature Salinity Intervals of water quality measurement	 not reported not reported not reported not reported not reported not reported not reported not reported not reported not reported 22°C not reported not reported
Number of replicates/groups: negative control: 0.004% ethanol treated ones:	3 replicates
Number of organisms per replicate /groups:	30 larvae per replicate
Biomass loading rate	30 larvae/4 L
Test concentrations: nominal:	 0.1 and 25 µg/L
Solvent (type, percentage, if used)	0.0036% ethanol in 10% Holtfreter's solution Holtfreter's medium:
Lighting	12 hrs light, 12 hrs dark
Feeding	Purina rabbit chow *ad libitum* (Purina Mills, St. Louis, MO)
Recovery of chemical Level of Quantitation Level of Detection	 detection limit: 0.1 µg/L

Parameter	Details
Positive control {if used, indicate the chemical and concentrations}	not reported
Other parameters, if any	

2. Observations:

Table 2: Observations

Criteria	Details
Parameters measured including the sublethal effects/toxicity symptoms	mortality, time to metamorphosis, weight and length at metamorphosis, sex based on gross morphology (all animals) and histology (9 females per treatment and on all males).
Observation intervals	not reported
Were raw data included?	No
Other observations, if any	

Field study sites were initially selected based on atrazine sales data and were located between 39°N and 43°N latitude. Counties with less than 0.4 kg/km^2 atrazine use were chosen as potential control sites, while areas with > 9.3 kg/km^2 atrazine use were chosen as potential atrazine-exposed sites. Sampling began in Utah on July 15, 2001 and moved eastward. Sampling stopped at the Iowa-Illinois border because *R. pipiens* populations were reportedly low or threatened in Illinois and Indiana.

A total of 8 sites (4 control and 4 "atrazine-contaminated") were sampled with 100 frogs collected at each site. Small frogs were intentionally collected in order to sample newly metamorphosed animals. Animals were immediately euthanized and fixed in Bouin's for 48 hours and preserved in 70% ethanol.

Back in the lab animals were measured, sex determined and histological analysis conducted on the gonads of 20 males from each site and a subset of females from each site.

Water samples (100 ml) were collected at each site in chemical-free glass jars (Fisher Scientific Co., Houston, TX) and frozen on dry ice immediately upon collection. Atrazine levels were determined by liquid chromatography/mass spectrophotometry (PTRL West, Inc., Hercules, CA); duplicate samples were analyzed using gas chromatography with a nitrogen phosphorous detectro (EPA method 507; Hygenic Laboratory University of Iowa, Iowa City, Iowa). In addition to parent atrazine, diaminochlorotriazine (DAC), deisopropylatrazine (DIA), deethylatrazine (DEA), two other triazines (simazine and hexazinone) and two other herbicides (diuron and norfluraone) were analyzed at all sites. The following pesticides were analyzed for water collected in Utah, Wyoming and Nebraska:: metolachlor, alachlor, glyphosate, metalaxyl, nicosulfuron, propiconazole, β-cyfluthrin, λ-cyhalothrin, and tebupirimphos.

II. RESULTS and DISCUSSION:

Laboratory Study

Control and atrazine-treated animals were sexually differentiated at metamorphosis, but 36% and 12% of the males treated with atrazine at 0.1 and 25 µg/L, respectively, suffered form gonadal dysgenesis (under-developed testes with poorly structured, closed lobules and low to absent germ cells.) Further, 29% of the 0.1 µg/L and 8% of the 25 µg/L animals displayed varying degrees of sex reversal. Testicular lobules of sex-reversed males contained oocytes, and males that metamorphosed later contained large numbers of oocytes. Males that appear to have undergone complete sex reversal had gonads completely filled with oocytes. In 2 males, oocytes were vitellogenic making the oocytes observable by gross morphology. Control males "never" contained testicular oocytes, although 2 control males contained 2 - 3 degenerating extragonadal oocytes (not in lobule) and a single male showed gonadal dysgenesis.

Field Study

None of the collection sites were atrazine-free (**Table 3**); however, atrazine residues were not reported at site 7. Except for metalochlor at site 5 (York County NE), pesticides were not found at any of the sites. Testicular oocytes were identified in males from 7 out of 8 collection sites. All sites with atrazine levels exceeding 0.2 µg/L had males that displayed sex-reversal similar to those abnormalities induced by atrazine in the lab. The highest incidence (92%) and most advanced cases of hermaphroditism were observed in animals collected from the North Platte River in Wyoming (site 3).

Table 3. Sample site, date, locality, description and atrazine residues for American leopard frog (*Rana pipien*) collections (source: Hayes *et al.* 2002).

Site	Date	State	County	Altitude (m)	Source	Habitat	Atrazine µg/L
1	7/15/01	Utah	Juab	1500	Pond	Grazeland	0.1
2	7/17/01	Utah	Cache	1555	Pond	Golf Course	0.2
3	7/19/01	Wyoming	Carbon	1952	river	Wildlife Area	0.2
4	7/23/01	Nebraska	Cherry	1031	Pond	Prarie	0.3
5	7/22/01	Nebraska	York	480	Ditch	Corn Field	0.8[1]
6	7/26/01	Iowa	Polk	252	Ditch	Corn Field	6.7
7	7/28/01	Iowa	Polk	246	Marsh	Wildlife Area	NA [2]
8	7/28/01	Iowa	Clinton	211	Stream	River Valley	0.5

[1] according to report figure, this value is more likely 8.0 µg/L.
[2] inconsistent results, one lab reported below level of detection

According to the authors, atrazine exposure disrupted gonadal development in exposed larvae as evidenced by poorly developed testicular tubules and reduced germ cells (gonadal dysgenesis) and oocytes developing in testes (testicular oogenesis); in a few cases, oocytes were vitellogenic.

F. REVIEWER'S COMMENTS:

This study is useful in identifying a potential hazard to amphibians and presents information on measurement endpoints, such as gonadal deformities. The study, however, does not show a clear dose response that demonstrates a causal relationship between atrazine exposure and developmental effects. The study authors suggested that the enhanced response at lower doses was consistent with low-dose effects reported for other endocrine-disrupting chemicals, but the data did not show a clear pattern of response. In a previous study using *Xenopus* (Hayes *et al.* 2002), there appeared to be a threshold effect for testicular abnormalities at 0.1 µg/L. The response, though, appeared to remain steady across increasing concentrations of atrazine with 16 - 20% of the males exhibiting gonadal abnormalities. Additional information is needed to support the authors conclusion that of an "inverted-U" (parabolic) dose response curve.

Access to the raw data supporting this study would also help the Agency verify the study's conclusions regarding time to metamorphosis, growth, and gonadal development. Although the study authors report that in some cases animals exhibited a mix of testicular and ovarian tissue and that the oocytes were vitellogenic, *i.e.*, contained yolk, they provide no evidence that any differential staining was done to verify that the oocytes were indeed vitellogenic. To better support their argument, a stain capable of differentiating phospholipids or glycolipids should have been used. The authors go on to state that induction of vitellogenesis in males would be consistent with upregulation of aromatase resulting in the increased production of endogenous estrogen. Upregulation of aromatase, according to the authors would also account for the failure to induce spermatogenesis (demasculinization) and induction of oocyte growth (feminization). The authors note that testicular oogenesis was not observed in any of the control animals nor in any of the *R. pipiens* routinely studied in their laboratories.

In this study, total atrazine residues (including atrazine degradates) are presented in graphical form and required extrapolation. According to this graph, parent atrazine residues (0.2 µg/L) at site 3 (highest incidence and most advanced cases of hermaphroditism) were similar to atrazine residues (0.2 µg/L) at site 2 (second lowest incidence of hermaphroditism. At site 4 (second highest incidence of testicular oogenesis), atrazine residues (0.3 µg/L) were relatively similar to site 3; however the atrazine degradates DEA, DIA and DAC resulted in total residues of approximately 1.2 µg/L. The highest residues of atrazine and its degradates occurred at site 5 [the study reports residues at 0.8 µg/L, but , the figure suggests that the reported value is actually 8 µg/L]. This site had the third highest rate of gonadal abnormalities, and the abnormalities were primarily gonadal dysgenesis rather than hermaphroditism. The second highest site (site 6) for atrazine residues (6.7 µg/L) corresponded to the third lowest incidence of gonadal abnormalities. Although the study showed gonadal effects in frogs, there was no clear pattern of response. Atrazine residues were not reported at Site 7 , and at least one of the two labs measuring atrazine reported that residues were below the level of detection; however, testicular oogenesis was reported in roughly 18% of the males examined.

In its evaluation, the Agency had questions concerning the use of ethanol as a co-solvent Atrazine concentrations used in the exposures are less than the 30 mg/L solubility limit of atrazine, and a co-solvent should not have been necessary . Previous studies conducted by the authors (personal communication: Tyrone Hayes 2002) have used dihydrotestosterone and 17-β estradiol as positive controls and required ethanol as a co-solvent. The current study, though, did not use these steroids, and a co-solvent should not have been needed.

EPA also needs more specific information concerning the methods used to determine the presence of vitillogenic oocytes. To better support their argument, a stain capable of differentiating phospholipids or

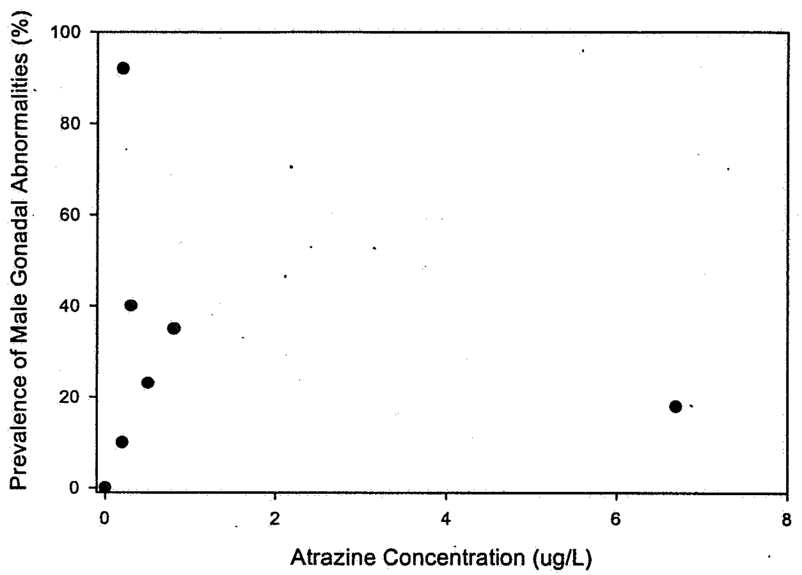

Figure 1. The prevalence of gonadal abnormalities in seven sites plotted against measured atrazine concentrations. One site is omitted because of a lack of atrazine analysis. Prevalence data are estimated from graphical representations of the data. This analysis also assumes that atrazine concentrations at site 5 are 0.8 ug/L, although figure 11A of the publication indicates that it was actually 8.0 ug/L.

glycolipids should be be used. In this study, the authors state that induction of vitellogenesis in males would be consistent with upregulation of aromatase resulting in the increased production of endogenous estrogen. Upregulation of aromatase, according to the authors would also account for the failure to induce spermatogenesis (demasculinization) and induction of oocyte growth (feminization). At the same time, the authors note that testicular oogenesis has not been observed in any of the control animals nor in any of the *R. pipiens* routinely studied in their laboratories.

Small frogs were intentionally sampled to assure that animals had recently metamorphosed implying that measured atrazine residues may have been reflective of exposure conditions during larval development. Additional information concerning the weights and age of the animals is needed to support this contention. Actual exposure conditions may have been considerably different than those reported in the study.

Although the researchers conducted pesticide residue analysis for chemicals that were reportedly used in the watershed, the scope of chemical analyses was on a site- by- site basis. As the authors noted, even atrazine was present where it was not used ,and it is likely that other chemical contaminants may have been present. Additional information on the chemical profile and morphoedapic characteristics of the sites is needed in order to compare the study sites.

The relationship of the potential atrazine exposure at the critical developmental stages is unknown because the surface water sampling was conducted during the post-metamorphic period. When the prevalence of male gonadal abnormalities is plotted as a function of atrazine concentration, there is no clear relationship (**Figure 1**). Additional data would help clarify this problem.

The Agency also needs clarifying data concerning the number of males and females collected at each site. Presumably at least 20 males were analyzed histologically, but it is unclear how these organisms were selected. If they were selected based on gross observations, then there is a strong possibility that the sample set was biased. Because a subset of organisms was selected and prevalence calculations were based on this selection, the subset should have been selected randomly.

In the laboratory study, 30 organisms per replicate with 3 replicates were exposed per treatment level. The prevalence of gonadal abnormalities was evidently based on combining the data of the replicates. It would be useful to understand the variance among the replicates and whether responses in individual tanks are driving the analysis. The Agency also needs data that indicates the distribution of males and females in the study and the sample sizes to gauge the robustness of the analyses. A further note concerning the laboratory study is the use of 30 organisms with an estimated maximal weight of 2.5 g in 4 liters of test solution. These conditions would result in a loading rate of 18.8 g/L, which is 37 times the recommended loading rate for static renewal toxicity tests.

The highest prevalence of gonadal abnormalities was observed at the site in Wyoming. Is it possible that there is a genetic component to this phenomenon? The authors state that in 7,000 organisms collected from four different states, no gonadal abnormalities [of the type described in the study] have ever been noted. Given the ubiquitous nature of atrazine contamination suggested in the paper, it is hard to imagine that these were all from atrazine-free environments, particularly in Wisconsin. If it is, in fact, true, then these data should have been published, as they would have provided great weight to the analysis.

As the study authors noted, the ecological relevancy of these findings is unclear because the researchers did not have problems in locating frogs. Further information is needed to determine if gonadal abnormalities impair the reproductive success, growth, and survival of amphibians.

H. REFERENCES:

Hayes, T. B., A. Collins, M. Lee, M. Mendoza, N. Noriega, A. A. Stuart, A. Vonk. 2002. Hermaphroditic, demasculinized frogs after exposure to the herbicide atrazine at low ecologically relevant doses. Proceedings of the National Academy of Science, 99 (8): 5476 - 5480.

Nieuwkoop, P. D. and J. Faber. 1994. Normal table of *Xenopus laevis* (Daudin). North-Holland Publishing Company, Amsterdam.

Data Evaluation Report on Response of the Amphibian Tadpole (*Xenopus laevis*) to Atrazine during Sexual Differentiation of the Ovary

EPA MRID Number: None

Data Requirement:
EPA DP Barcode None

EPA MRID Not Assigned
EPA Guideline Open Literature

Test material: **Purity:** 99%
Common name: Atrazine
Chemical name: IUPAC
CAS name 6-chloro-N-ethyl-N'-(1-methylethyl)-1,3,5-triazine-2,4-diamine
CAS No. 1912-24-9
synonyms
EPA PC Code: 80803

Thomas M Steeger

Primary Reviewer: Thomas M. Steeger, Ph.D., Senior Biologist **Date:** April 30, 2003
Environmental Fate and Effects Division, ERB 4,
U. S. Environmental Protection Agency

Joseph E. Tietge

Secondary Reviewer(s): Joseph E. Tietge, M.S., Research Aquatic Biologist **Date:** 5/1/03
Mid-Continent Ecology Division, National Health and Environmental Effects Research Laboratory (Duluth), U. S. Environmental Protection Agency

Stephanie R. Irene

Stephanie Irene, Ph.D., Senior Advisor **Date:** 5/6/03
Environmental Fate and Effects Division, ERB 3, U. S. Environmental Protection Agency

Mary Frankenberry

Mary Frankenberry, Senior Statistician **Date:** 5/5/03
Environmental Fate and Effects Division, ERB 3, U. S. Environmental Protection Agency

EPA PC Code 080803

TJS 06/02/03

Date Evaluation Completed: ~~04~~/30/2003

CITATION: Tavera-Mendoza, L., S. Ruby, P. Brousseau, M. Fournier, D. Cyr and D. Marcogliese. 2001. Response of the amphibian tadpole (*Xenopus laevis*) to atrazine during sexual differentiation of the ovary. Environmental Toxicology and Chemistry 21: 1264 - 1267.

EXECUTIVE SUMMARY:

In an effort to examine the effects of atrazine on gonadal differentiation during larval tadpole development of the female African clawed frog (*Xenopus laevis*), larvae (Nieuwkoop-Faber Stage 56) were exposed to mean-measured concentrations of atrazine at 18 μg/L (nominal: 21 μg/L) under static conditions for 48 hours. Animals were fasted during the 48-hour test. The frequency of occurrence of primary oogonia was significantly ($p < 0.05$) lower in atrazine-exposed (43.7%) tadpoles relative to controls (74%); however, the frequency of occurrence of secondary oogonia was significantly ($p < 0.05$) higher in atrazine-exposed (36%) tadpoles compared to controls (23%). The incidence of atretic primary and secondary oogonia was significantly higher ($p < 0.05$) in atrazine-exposed ovaries (20.2%) relative to control (2%). Furthermore, sections of the pituitary revealed no histological evidence that the pituitary was actively secreting hormones. The authors concluded that atresia could reduce the reproductive capacity of the tadpole because primary germ cells provide oocytes for all the subsequent cycles of oogenesis in th reproductive life of the frog. Although the authors speculated that atrazine may be affecting aromatase activity, they were unable to provide a mechanism by which conversion of androgens to estrogen could affect the endpoints measured in the study. In addition, there were no data available to indicate an endogenous source of estrogen in the *Xenopus* gonad during this stage of development.

This is a nonguideline study and does not provide the level of detail in terms of methodology and results that EPA typically uses to evaluate studies. This study provides useful data on the effects of atrazine on ovarian development, but it does not establish a dose-response relationship because only one concentration of atrazine was tested. The measurement endpoints are relatively undocumented in *X. laevis,* and it is unclear how the effects discussed in this paper may impact reproductive capacity, growth and/or survival of affected animals even though the authors stated that atresia could reduce the reproductive capacity of the tadpoles. The effects observed in the ovaries after only 48 hours of exposure are significant, but the study has not been repeated.

I. MATERIALS AND METHODS

GUIDELINE FOLLOWED: Nonguideline Study
COMPLIANCE: Not conducted under full Good Laboratory Practices

A. MATERIALS:

1. Test Material Atrazine

Description:

Lot No./Batch No. : Not reported

Purity: 99% (Sigma Chemical Co., St. Louis, MO)
Stability of compound under test conditions: Not reported

Storage conditions of test chemicals:_ Not reported

2. Test organism:

Species: African clawed frog (*Xenopus laevis*)

Age at test initiation: Niewkoop-Faber stage 54 tadpoles just prior to gonadal differentiation.
Weight at study initiation (mean and range): Not reported
Length at study initiation (mean and range): Not reported

Source: Xenopus 1 (Dexter, MI, USA)

B. STUDY DESIGN:

Objective: To examine the effects of atrazine on gonadal differentiation during larval tadpole development of the female African clawed frog.

1. Experimental Conditions

a) **Range-finding Study**: Exposure concentration based on atrazine residues recently reported in St. Lawrence Rive Valley region of Quebec, CA
b) **Definitive Study**

Table 1 . Experimental Parameters

Parameter	Details
Acclimation: period: conditions: (same as test or not) Feeding: Health: (any mortality observed)	Progeny were collected from 10 malesand 10 females and were acclimated for one week 100-L glass aquaria containing 15-L dechlorinated Montreal tap water at 21°C on a 12:12 (light:dark) cycle Fed specially prepared tadpole diet (Boreal, St. Catherines, Ontario, CA) twice per week during acclimation
Duration of the test	48 hours starting at NF stage 56
Test condition static/flow- through Type of dilution system for flow-through method. Renewal rate for static renewal	 static NA NA
Aeration, if any	aerated
<u>Test vessel</u> Material: (glass/stainless steel) Size: Fill volume:	 glass 100-L 15-L fill volume
Source of dilution water Quality:	City of Montreal (Canada) tap water

Parameter	Details
Water parameters: Hardness pH Dissolved oxygen Total organic carbon Particulate matter Ammonia Nitrite Metals Pesticides Chlorine Temperature Salinity Intervals of water quality measurement	 128 mg/L pH = 7.8 90% saturation not reported not reported not reported not reported not reported not reported not reported 21 ± 0.05°C not reported not reported
Number of replicates/groups: negative control: treated ones:	2 replicates per treatment
Number of organisms per replicate /groups:	16 tadpoles/replicate
Biomass loading rate	16 tadpoles/15 L
Test concentrations: nominal:	 0 and 21 µg/L
Solvent (type, percentage, if used)	none
Lighting	12 hours dark, 12 hours light
Feeding	no feeding during 48-hr exposure
Recovery of chemical Level of Quantitation Level of Detection	Water samples were collected at 0 and 48 hours for atrazine analysis by HPLC/MS (Bodycote Technitrol (Point Claire, PQ, CA). Cyanazine and simazine concentrations were also measured.
Positive control {if used, indicate the chemical and concentrations}	

Parameter	Details
Other parameters, if any	

2. Observations:

Table 2: Observations

Criteria	Details
Parameters measured including the sublethal effects/toxicity symptoms	
Observation intervals	
Were raw data included?	
Other observations, if any	Gonad-kidney was prepared for histological analysis. Brain along with attached pituitary were stained to determine if cells in the pituitary were actively secreting hormones. Frequency and occurrence of primary and secondary oogonia and atresia (process whereby developing eggs are resorbed in the ovary) were measured in each ovary.

Statistical analyses employed the nonparametric analog of an unpaired t-test, the Mann Whitney U test.

II. RESULTS and DISCUSSION:

Exposure was measured at 0 and 48 hours. Control values contained atrazine at < 0.05 µg/L,while the nominal 21 µg/L tank contained atrazine at 18 µg/L. Therefore, mean-measured atrazine concentrations were roughly 87% of nominal.

The frequency of occurrence of primary oogonia was significantly ($p < 0.05$) lower in atrazine-exposed (43.7%) tadpoles relative to controls (74%); however, the frequency of occurrence of secondary oogonia was significantly ($p < 0.05$) higher in atrazine-exposed (36%) tadpoles compared to controls (23%). The incidence of atretic primary and secondary oogonia was also significantly higher ($p < 0.05$) in atrazine-exposed ovaries (20.2%) relative to controls (2%).

Sections of the pituitary revealed no histological evidence that the pituitary was actively secreting hormones. Only inactive chromophobes were present in the pituitary of both control and treated tadpoles.

The study authors concluded that their findings suggest that exposure of tadpoles to atrazine at 21 μg/L for 48 hrs during sexual differentiation of the ovary resulted in a 20% decline in primary and secondary oogonia through the process of atresia compared to a 2% decline in the controls. Although the formation of secondary oogonia appears to have increased in atrazine-treated females, 20% of those formed underwent resorption before progressing to the primary oocyte stage. Because these primary germ cells provide oocytes for all the subsequent cycles of oogenesis in th reproductive life of the frog, atresia could reduce the reproductive capacity of the tadpole. The authors speculated that the observed effects are probably not a result of an estrogenic effect of atrazine, because an endogenous source of estrogen was not present in the *Xenopus* eggs during this stage of development. Additionally they believed that atrazine does not affect the hypothalmic-pituitary-gonadal axis. The presence of chromophores in the adenohypophysis of the tadpole suggests that the pituitary was inactive at the time of exposure to atrazine. They suggested, however, that atrazine may be acting on aromatase activity, but they did not indicate how the up-regulation of aromatase activity might affect atresia. The authors discussed a similar study (Tavera-Mendoza et al. 2001) which was conducted in their lab, where atrazine treatment increased the rate of testicular resorption and reduced the number of spermatogonial cell nests thus reducing the overall reproductive capacity of the male for the life of the frog. The effects observed in testes could be related to an up-regulation of aromatase activity. The authors claimed that the potential for extrapolating their results to other vertebrates is high because testosterone and estrogen receptor–ligand binding sites are highly conserved as well as the mRNA nucleotide sequence for both 5 -reductase and aromatase.

F. REVIEWER'S COMMENTS:

According to Nieuwkoop-Faber, stage 54 corresponds to age ± 26 days with a length corresponding to 58 - 65 mm.. Stage 56 corresponds to age ±38 days with lengths ranging from 70 - 100 mm.

This study used dechlorinated tap water. EPA recommends the use of dechlorinated water unless chlorine residue analysis is conducted to verify that the water is indeed dechlorinated. Additionally, a limited number (pH and water temperature) of water quality parameters were reported.

A stock solution of atrazine was prepared by dissolving 0.15 g atrazine in 1L distilled water and ultrasonicating it for 6 hours in an ice bath. This is relatively harsh treatment for a compound. Stock solution: 0.1485 mg a.i./mL (21 mL) = 0.3119 mg a.i./15L = 20.79 μg a.i./L treatment solution.

Although cyanazine and simazine concentrations were determined, no data were provided on the results of these assessments.

This study provides useful data on the effects of atrazine on ovarian development, but it does not establish a dose-response relationship because only one concentration of atrazine was tested.

The effects observed in the ovaries after only 48 hours of exposure are significant, but has not been repeated.

H. REFERENCES:

Hayes, T. B., A. Collins, M. Lee, M. Mendoza, N. Noriega, A. A. Stuart, A. Vonk. 2002. Hermaphroditic, demasculinized frogs after exposure to the herbicide atrazine at low ecologically relevant doses. Proceedings of the National Academy of Science, 99 (8): 5476 - 5480.

Nieuwkoop, P. D. and J. Faber. 1994. Normal table of *Xenopus laevis* (Daudin). North-Holland Publishing Company, Amsterdam.

Data Evaluation Report on Response of the Amphibian Tadpole (*Xenopus laevis*) to Atrazine During Sexual Differentiation of the Testes

EPA MRID Number: None

Data Requirement:

EPA DP Barcode None

EPA MRID Not Assigned
EPA Guideline Open Literature

Test material: **Purity:** 99%

Common name Atrazine
chemical name: IUPAC
CAS name 6-chloro-N-ethyl-N'-(1-methylethyl)-1,3,5-triazine-2,4-diamine
CAS No. 1912-24-9
synonyms
EPA PC Code: 80803

Thomas M Steeger

Primary Reviewer: Thomas M. Steeger, Ph.D., Senior Biologist **Date:** April 30, 2003
Environmental Fate and Effects Division, ERB 4, U. S. Environmental Protection Agency

Joseph E. Tietge

Secondary Reviewer(s): Joseph E. Tietge, Research Aquatic Biologist **Date:** 5/1/03
Mid-Continent Ecology Division, National Health and Environmental Effects Research Laboratory (Duluth), U. S. Environmental Protection Agency

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Mary Frankenberry

Mary J. Frankenberry, Senior Statistician **Date:** 5/1/03
Environmental Fate and Effects Division, ERB 3, U. S. Environmental Protection Agency

EPA PC Code 080803

Date Evaluation Completed: 05/01/2003

CITATION: Tavera-Mendoza, L., S. Ruby, P. Brousseau, M. Fournier, D. Cyr and D. Marcogliese. 2001. Response of the amphibian tadpole (*Xenopus laevis*) to atrazine during sexual differentiation of the testis. Environmental Toxicology and Chemistry 21: 527 - 531.

EXECUTIVE SUMMARY:

In an effort to examine the effects of atrazine on gonadal differentiation and reproductive impairments, male African clawed frog larvae (Nieuwkoop-Faber Stage 56) were exposed to mean-measured concentrations of atrazine at 18 µg/L (nominal: 21 µg/L) under static conditions for 48 hours. Animals were fasted during the 48 hours test. Total testicular volume decreased significantly ($p = 0.004$) from 0.026 ± 0.003 mm^3 in controls to 0.01 ± 0.001 m^3 in atrazine-treated tadpoles representing a 57% decrease after 48 hours of exposure. The number of spermatogonial cell nests decreased significantly ($p < 0.001$) from an mean of 242.4 ± 35.7 in controls to 72.9 ± 21.8 in atrazine-exposed tadpoles representing a 70% reduction. The number of nursing cells declined significantly ($p < 0.001$) from a mean of 9.62 ± 0.17 in controls to 2.35 ± 0.36 in atrazine treated tadpoles. Testicular resorption was observed in 70% of the male tadpoles exposed to atrazine relative to controls; failure of full development of the testis (aplasia) was observed in 10% of the testes examined. Histological examination of the pituitary suggested that tissues were actively secreting hormones based on the absence of chromophores.

This study provides useful information on hazard identification and measurement endpoints, such as gonadal abnormalities. However, the study does not provide sufficient information to establish a dose-response relationship because only one concentration of atrazine was tested. Other information which is needed to more fully interpret the significance of this study include: documentation of the measurement endpoints, elaboration on the ecological relevancy of the measurement endpoints, and clarification of the number of exposed animals.

I. MATERIALS AND METHODS

GUIDELINE FOLLOWED: Nonguideline Study
COMPLIANCE: Not conducted under full Good Laboratory Practices

A. MATERIALS:

1. Test Material Atrazine

Description:

Lot No./Batch No. : Not reported

Purity: 99% (Sigma Chemical Co., St. Louis, MO)
Stability of Compound Under Test Conditions: Not reported

Storage conditions of test chemicals:_ Not reported

2. Test organism:

Species: African clawed frog *(Xenopus laevis)*

Age at test initiation: Niewkoop-Faber stage 54 tadpoles just prior to gonadal differentiation.
Weight at study initiation: (mean and range) not reported
Length at study initiation: (mean and range) not reported

Source: Xenopus 1 (Dexter, MI, USA)

B. STUDY DESIGN:

Objective: To examine the effects of atrazine on gonadal differentiation and reproductive impairments.

1. Experimental Conditions

a) **Range-finding Study**: Exposure concentration based on atrazine residues recently reported in St. Lawrence Rive Valley region of Quebec, CA
b) **Definitive Study**

Table 1 . Experimental Parameters

Parameter	Details
Acclimation: period: conditions: (same as test or not) Feeding: Health: (any mortality observed)	1 week 100-L glass aquaria containing 15-L dechlorinated Montreal tap water at 21°C on a 12:12 (light:dark) cycle Fed specially prepared tadpole diet (Boreal, St. Catherines, Ontario, CA) twice per week during acclimation
Duration of the test	48 hours starting at NF stage 56
Test condition static/flow through Type of dilution system- for flow through method. Renewal rate for static renewal	 static NA NA
Aeration, if any	aerated
Test vessel Material: (glass/stainless steel) Size: Fill volume:	 glass 100-L 15-L fill volume
Source of dilution water Quality:	City of Montreal (Canada) tap water
Water parameters: Hardness pH Dissolved oxygen Total Organic carbon Particulate Matter Ammonia Nitrite Metals Pesticides Chlorine Temperature Salinity Intervals of water quality measurement	 not reported pH = 7.6 not reported not reported not reported not reported not reported not reported not reported not reported 21°C not reported not reported

Parameter	Details
Number of replicates/groups: negative control: treated ones:	2 replicates per treatment
Number of organisms per replicate /groups:	16 tadpoles/replicate
Biomass loading rate	16 tadpoles/15 L
Test concentrations: nominal:	0 and 21 µg/L
Solvent (type, percentage, if used)	none
Lighting	12 hours dark, 12 hours light
Feeding	no feeding during 48-hr exposure
Recovery of chemical Level of Quantitation Level of Detection	Water samples collected at 0 and 48 hours for quantitation by HPLC/MS
Positive control {if used, indicate the chemical and concentrations}	
Other parameters, if any	

2. Observations:
Table 2: Observations

Criteria	Details
Parameters measured including the sublethal effects/toxicity symptoms	
Observation intervals	
Were raw data included?	
Other observations, if any	Gonad-kidney prepared for histological analysis. Brain along with attached pituitary stained to determine if cells in the pituitary were actively secreting hormones. Index of total testicular volume , estimated number of spermatogonial cell nests and nurse cell integrity measured in each testis.

For the three testicular parameters, data were ranked to satisfy the assumption of a normal distribution and then subjected to one-way ANOVA followed by Tukey's test (SPSS10 program; SPSS, Chicago, IL

II. <u>RESULTS</u> and <u>DISCUSSION</u>:

Exposure measured at 0 and 48 hours. Control values contained atrazine at < 0.05 µg/L while the nominal 21 µg/L tank contained atrazine at 18 µg/L. Therefore, mean-measured atrazine concentrations were roughly 87% of nominal.

Total testicular volume decreased significantly ($p = 0.004$) from 0.026 ± 0.003 mm3 in controls to 0.01 ± 0.001 m3 in atrazine-treated tadpoles representing a 57% decrease after 48 hours of exposure. The number of spermatogonial cell nests decreased significantly ($p < 0.001$) from an mean of 242.4 ± 35.7 in controls to 72.9 $\pm$ 21.8 in atrazine-exposed tadpoles representing a 70% reduction. The number of nursing cells declined significantly ($p < 0.001$) from a mean of 9.62 ± 0.17 in controls to 2.35 ± 0.36 in atrazine treated tadpoles. Testicular resorption was observed in 70% of the male tadpoles exposed to atrazine relative to controls; failure of full development of the testis (aplasia) was observed in 10% of the testes examined.

Histological sections of the pituitary revealed only undifferentiated chromophobes in both control and treated tadpoles. No evidence was found of chromophobes, indicating that the pituitary was actively secreting hormones.

The study authors conclude that their findings suggest that the developing germ cells in tadpole testes during testicular differentiation are highly sensitive to atrazine and that because these are primary sources of germ cells for the life of the organism, the effects would not be transient and recovery could not occur. The authors suggest that atrazine may disrupt sexual differentiation by altering testosterone metabolism through up-regulation of CYP19 and consequent increased activity of aromatase. Increased aromatase activity could increase transformation of testosterone to estrogen. Alternatively they suggest that atrazine could directly block testosterone and/or dihydrotestosterone at the receptor level. The authors claim that the potential for extrapolating their results to other vertebrates is high given how highly conserved testosterone and estrogen receptor–ligand binding sites are as well as the mRNA nucleotide sequence for both 5α-reductase and aromatase.

This is a nonguideline study and does not provide the level of detail in terms of methodology and results that EPA typically uses to evaluate studies. The methodology section contains some inconsistencies in terms of the number of animals used in the study. This study provides data on the effects of atrazine on testicular development; however, with only a single concentration of atrazine tested, it isn't possible to characterize a dose response. The measurement endpoints are relatively undocumented in *Xenopus laevis* and it is unclear how the effects discussed in this paper may impact reproductive capacity, growth and/or survival of affected animals. Taken at face value, the effects observed in the testes after only 48 hours of exposure are rather remarkable. However, the scope of this study is quite limited and is unrepeated, even within this laboratory.

F. REVIEWER'S COMMENTS:

According to Nieuwkoop-Faber, stage 54 corresponds to age ± 26 days with a length corresponding to 58 - 65 mm.. Stage 56 corresponds to age ±38 days with lengths ranging from 70 - 100 mm.

According to the methods section, there are two replicates per treatment and there are two treatments (0 and 21 µg/L); therefore there are 4 tanks in total with 16 tadpoles per tank. This should yield 64 tadpoles in the study; however the authors claim that the total number of tadpoles for the experiment was 48.

According to the study, the total number of males was 24 and the distribution of males in each of the tanks was 7 and 9 in the controls and 8 and 8 in the treated tanks (7+9+8+8= 32 males) not 24 males.

Study used dechlorinated tap water. EPA recommends the use of dechlorinated water unless chlorine residue analysis is conducted to verify that the water is indeed dechlorinated. Additionally, a limited number (pH and water temperature) of water quality parameters are reported.

Stock solution of atrazine prepared by dissolving 0.15 g atrazine in 1 L distilled water and ultrasonicating for 6 hours in an ice bath. This is relatively harsh treatment for a compound. Why not make up a more dilute solution? Stock solution: 0.1485 mg a.i./mL (21 mL) = 0.3119 mg a.i./15L = 20.79 µg a.i./L treatment solution.

While apparently substantial declines in testicular volume (57%) and spermatogonial cell nests (70%) occurred following exposure to atrazine 18 µg a.i./L and testicular resorption was observed in 70% of the male tadpoles exposed to atrazine relative to controls, no data are provided on the extent to which reproductive success would be impaired. With a single dose of atrazine tested, there is no way to determine whether the effect is dose-related. Also, the methodology section contained some inconsistencies on the actual number of frogs used in the study; the actual sample sizes are not clear. However, in general while sample reported sizes were not high, the treatments were replicated.

Taken at face value, the effects observed in the testes after only 48 hours of exposure are rather remarkable. However, the scope of this study is quite limited and is unreapeated, even within this laboratory. Unfortunately, the study does not include measurements on the gonads of organisms prior to the exposure.

H. REFERENCES:

Hayes, T. B., A. Collins, M. Lee, M. Mendoza, N. Noriega, A. A. Stuart, A. Vonk. 2002. Hermaphroditic, demasculinized frogs after exposure to the herbicide atrazine at low ecologically relevant doses. Proceedings of the National Academy of Science, 99 (8): 5476 - 5480.

Nieuwkoop, P. D. and J. Faber. 1994. Normal table of *Xenopus laevis* (Daudin). North-Holland Publishing Company, Amsterdam.