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

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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*)

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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

Parameter	Details
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
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. RESULTS and DISCUSSION:

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 = 8.9×10^2 fmol/h/mg protein) than in males (7.2×10^2 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. <u>REPORTED STATISTICS</u>:

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)	$3.5 \times 10^{-1*}$	6.8×10^{-2}	3.6×10^{-1}*	7.2×10^{-2}
17-β estradiol	$1.5 \times 10^{-1**}$	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. VERIFICATION OF STATISTICAL RESULTS: 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 20ºC, 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

NONPARAMETRIC COMPARISON OF FROG WEIGHT ACROSS TREATMENTS BY SEX 139

---------- 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

ANOVA FOR LENGTH OF FROGS ACROSS TREATMENT BY SEX 141

---------- 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

NONPARAMETRIC COMPARISON OF FROG LENGTHS ACROSS TREATMENTS BY SEX 149

---------- 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

ANOVA FOR WEIGHT OF FROGS ACROSS POSITIVE CONTROLS BY SEX 157

---------- 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

NONPARAMETRIC COMPARISON OF FROG WEIGHT ACROSS POSITIVE CONTROLS 161

---------- 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

NONPARAMETRIC COMPARISON OF FROG WEIGHT ACROSS POSITIVE CONTROLS 163

---------- 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

ANOVA FOR LENGTH OF FROGS ACROSS POSITIVE CONTROLS BY SEX 165

---------- 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

ANOVA FOR LENGTH OF FROGS ACROSS POSITIVE CONTROLS BY SEX 169

---------- 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

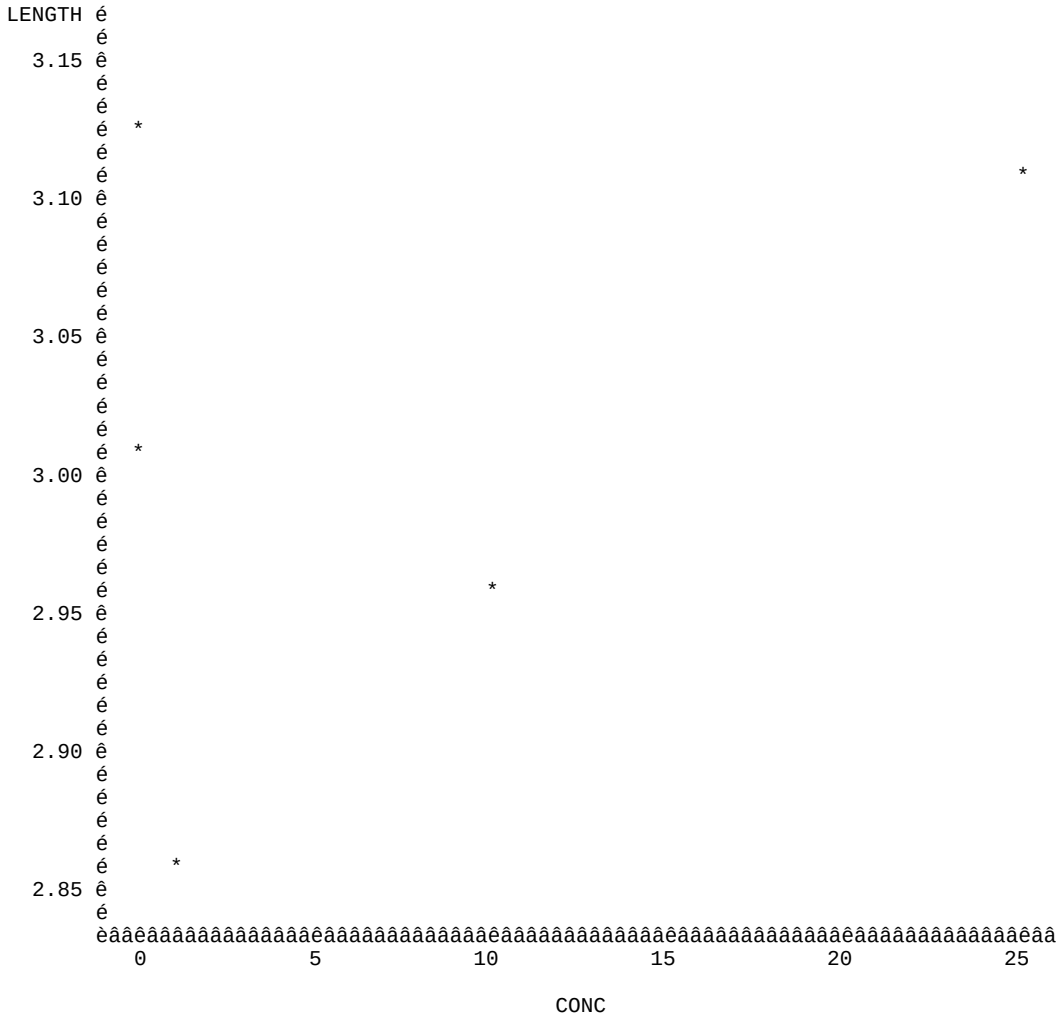
Median One-Way Analysis

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

AVERAGE LENGTH OF FROGS OVER TREATMENTS BY SEX 177

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

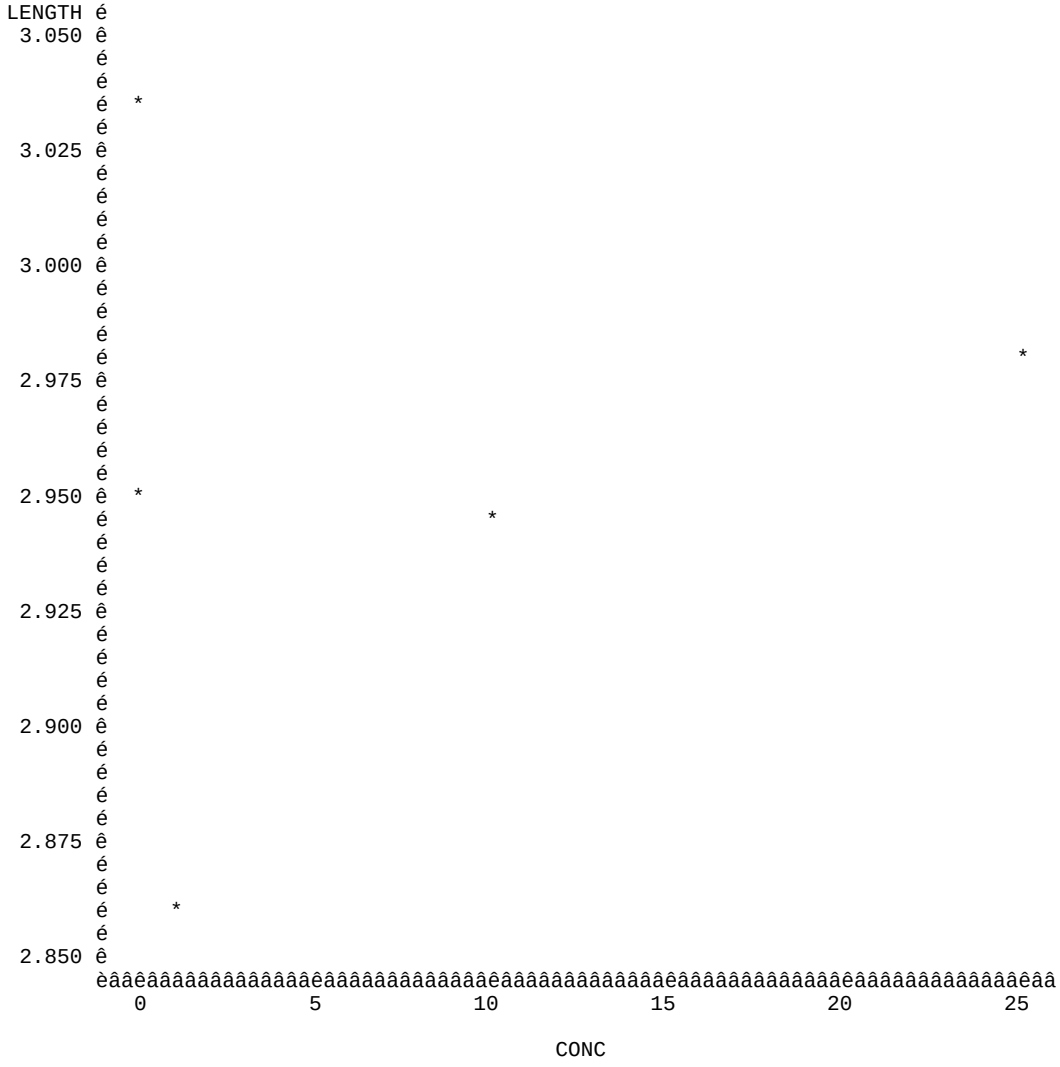
Plot of LENGTH*CONC. Symbol used is '*'.



AVERAGE LENGTH OF FROGS OVER TREATMENTS BY SEX 178

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

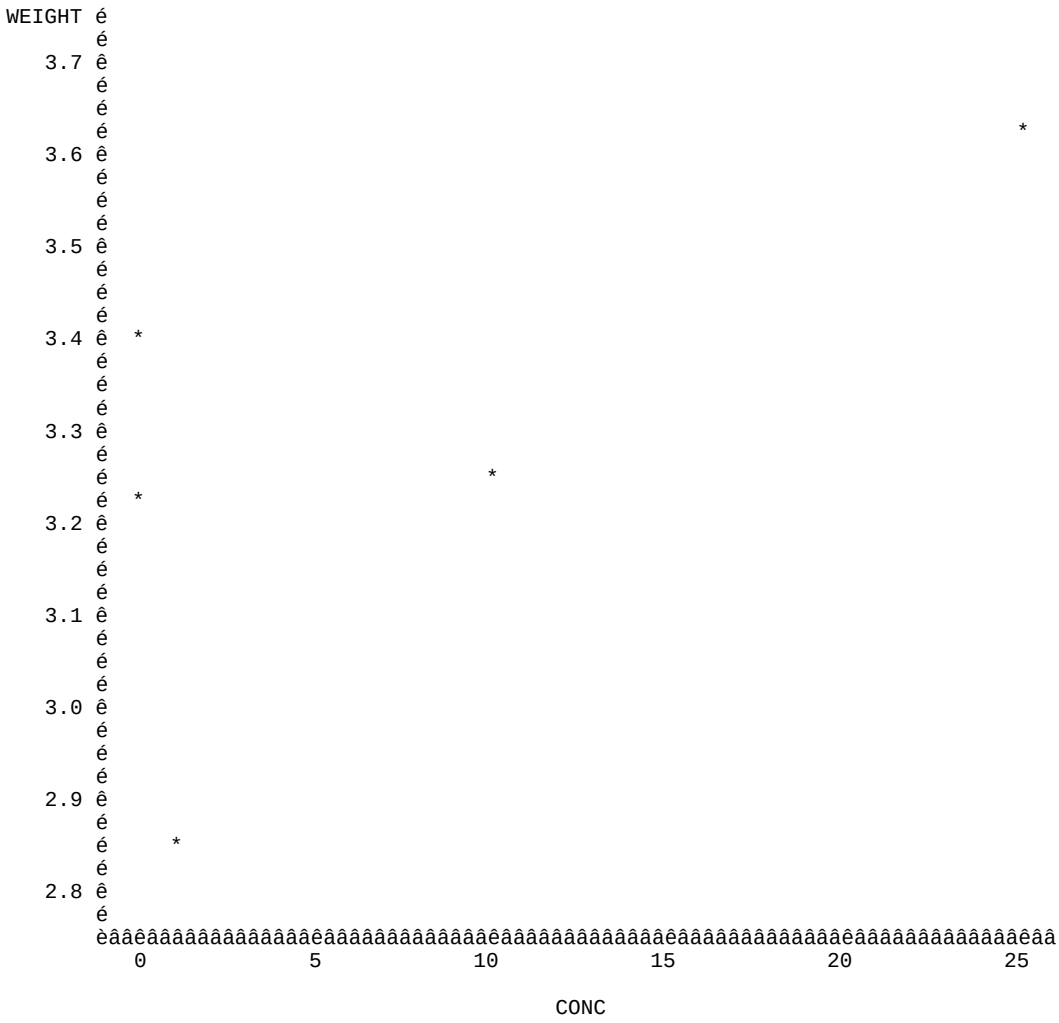
Plot of LENGTH*CONC. Symbol used is '*'.



AVERAGE WEIGHT OF FROGS OVER TREATMENTS BY SEX 179

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

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



AVERAGE WEIGHT OF FROGS OVER TREATMENTS BY SEX 180

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

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