White Paper on the Potential for Atrazine to Affect Amphibian Gonadal Development

In Support of an
Interim Reregistration Eligibility Decision
on Atrazine

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for Review and Comment
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EXECUTIVE SUMMARY

BACKGROUND

Under an amended consent decree entered in to conclude litigation between the Environmental Protection Agency (EPA) and the Natural Resources Defense Council (NRDC), the Agency issued an Interim Reregistration Eligibility Decision (IRED) for atrazine on January 31, 2003. The decree further stipulated that EPA would issue a revised IRED on October 31, 2003 that considers studies conducted prior to February 28, 2003 on the effects of atrazine on amphibians. After developing a paper that addressed the significance of the amphibian risk data, the Agency agreed, under the consent decree, to seek external peer review of its evaluation of these studies from a Federal Insecticide, Fungicide and Rodenticide Act (FIFRA) Scientific Advisory Panel (SAP). In June 2003, the SAP reviewed a White Paper prepared by EPA (USEPA 2003) that critically evaluated the collective available data from 17 laboratory and field studies, discussed the nature of remaining uncertainties in evaluating the potential effects of atrazine on amphibian development, and outlined the nature of future studies that could address these uncertainties.

In its 2003 White Paper, EPA concluded that while there was sufficient information to formulate a hypothesis that atrazine exposure can affect amphibian gonadal development, there was insufficient information to refute or confirm the hypothesis. Collectively, the available studies had deficiencies and uncertainties that limited their usefulness in interpreting potential atrazine effects. In response to these uncertainties, the Agency identified additional information necessary to evaluate the potential causal relationship between atrazine exposure and gonadal development in amphibians and the nature of any associated dose-response relationship. EPA proposed that future amphibian studies be conducted in a tiered approach. After reviewing EPA's analysis and recommendations, the SAP concurred that the Agency's review was thorough, the approaches and criteria were appropriate, and the conclusions were valid, given the data reviewed. The SAP also agreed that additional studies were warranted and that a tiered testing approach was appropriate (SAP 2003).

In November 2004, EPA issued a Data Call-in (DCI) Notice to Syngenta Crop Protection, Inc., and other atrazine registrants requiring the registrants to conduct a study consistent with the first tier of testing described in the 2003 White Paper. After the Agency and the registrant agreed

to a protocol for testing potential effects of atrazine on gonadal development of amphibians in April 2005, the registrant submitted to EPA in June 2007 its final report entitled "Response of Larval *Xenopus laevis* to Atrazine Exposure: Assessment of Metamorphosis and Gonadal Morphology. Consistent with the Tier 1 study proposed by EPA, the registrant's protocol and final report focused on the effects of atrazine alone on amphibian gonadal development.

OVERVIEW OF LABORATORY AND FIELD STUDIES

Before developing the 2007 White Paper, EPA searched the open literature for any studies published since the preparation of the 2003 White Paper (USEPA 2003). After identifying recent laboratory and field studies related to potential effects of atrazine on gonadal development in amphibians, EPA published a list of these studies in the Federal Register and solicited recommendations for additional studies from the public. Based on this literature search, a total of 19 new studies (**Table 1),** which were published in the open literature or submitted by the registrant, were reviewed. Ten were laboratory-based investigations and nine were field studies. Of the ten laboratory-based studies, two were the final versions of studies reviewed as interim reports for the 2003 SAP (USEPA 2003). All of the field studies were final versions of studies reviewed as interim reports for the 2003 SAP (USEPA 2003). Each study was individually evaluated with regard to the effects of atrazine alone on amphibian gonadal development. Furthermore, each of the studies was evaluated relative to experimental design, protocols and data quality assurance, strength of the cause-effect and/or dose-response relationship, and ecological relevancy of measured endpoints.

Laboratory Studies

Similar to what was discussed in the Agency's 2003 White Paper (USEPA 2003), laboratory studies addressed a variety of endpoints: time to metamorphosis, growth, gonadal abnormalities, sex ratios, laryngeal muscle area, plasma steroid concentrations, and aromatase activity. In evaluating the effects of atrazine on amphibian development, researchers tested a variety of frog species, including African clawed frogs (*Xenopus laevis*), the Northern leopard frog (*Rana pipiens*), and the green frog (*R. clamitans*). Not all species were used in every study. All of the studies, except one that was submitted by the registrant in response to the DCI, used static renewal exposures. Within individual studies the number of atrazine exposures ranged from only one concentration up to five different concentrations; in one study, atrazine concentrations spanned five orders of magnitude (0.01 to 100 ppb). In some cases, the nominal

atrazine concentrations were confirmed analytically. Although three studies (Hayes *et al*. 2006b; Orton *et al*. 2006; Sullivan and Spence 2003) reported effects of atrazine in combination with other chemicals, this review focused on the effects of atrazine alone. As recommended by the 2003 SAP (SAP 2003), this review also focused on gonadal development in amphibians and not other endpoints.

Only one of the laboratory studies, *i.e*., the study submitted by the registrant in response to the DCI, incorporated all of the design elements recommended by the Agency and the FIFRA SAP (SAP 2003) to address uncertainties identified in the Agency's 2003 White Paper (USEPA 2003). This submission represented the results of two separate studies conducted in two independent laboratories. Although both these studies contain limitations, *e.g.*, contamination of a subset of contols in one study, the overall design and conduct of the studies contain a high degree of quality control that allows them to be used in characterizing ecological risk. The results of these recently submitted studies show that atrazine did not have a consistent effect on time to or size at metamorphosis or on gonadal development in *X. laevis*.

The studies submitted in response to the DCI were the only laboratory studies that fully accounted for environmental and animal husbandry factors capable of influencing endpoints which the studies were attempting to measure. Other laboratory studies reviewed by the Agency did not fully account for environmental conditions that could influence relevant endpoints.

Field Studies

All of the field studies were reviewed as interim reports in the 2003 White Paper (USEPA 2003). The field studies, which include a mesocosm study, evaluated growth, gonadal deformities, sex ratios, plasma steroid concentrations, and aromatase activity in *X. laevis*, *R. clamitans*, bullfrogs (*R. catesbeiana*), and cane toads (*Bufo marinus*) in Florida, Indiana, Iowa, Michigan, and South Africa.

Similar to the conclusions regarding field studies discussed in the 2003 White Paper (USEPA 2003), the Agency concludes that the currently available field studies are of limited value because of the high variability in environmental conditions (*e.g.,* photoperiod, temperature, water quality) under which field-collected organisms lived, uncertainty as to amphibian developmental status and condition at the initiation of the studies, and an inability to relate the co-occurrence of atrazine with key developmental windows for the organisms under investigation. Consequently, EPA cannot determine whether the failure of several studies to show any

relationship between measured or predicted aqueous atrazine concentrations and developmental effects reflects the absence of a causal relationship or the limitations of the study methodologies. In addition, the actual or possible co-occurrence of additional chemical and/or non-chemical stressors confound attempts to attribute any observed responses to atrazine exposure.

CONCLUSIONS FROM LABORATORY AND FIELD STUDIES

The Agency has reviewed 36 open literature and registrant-submitted studies related to the potential effects of atrazine on gonadal development in amphibians. Overall, the weight-of-evidence based on these studies does not show that atrazine produces consistent, reproducible effects across the range of exposure concentrations and amphibian species tested. In laboratory studies where environmental and animal husbandry factors were controlled, atrazine exposures (0.01 – 100 μg/L) did not affect time to or size at metamorphosis, sex ratio, or gonadal development. While there were several effects on secondary gross and histological endpoints that were statistically significant, their relationship to apical endpoints of intersex and/or gonadal development effects is not considered relevant.

Because of the uncertainties associated with all the laboratory and field studies conducted prior to 2003, the 2003 SAP recommended that additional studies be conducted to determine if exposure to atrazine affects amphibian gonadal development. In their report, the SAP recommended using *X. laevis* as the test species as well as indigenous species. Because the SAP report did not identify what benefits the indigenous species would provide, the Agency concluded that testing with *X. laevis* would be sufficient for a Tier 1 study. Based on the recent nineteen studies reviewed, including the recently submitted DCI studies showing no effects of atrazine on amphibian gonadal development, the Agency has further concluded that the higher tiers of testing proposed in the 2003 White Paper (USEPA 2003) are not needed at this time.

Table 1. Summary of open literature and registrant-submitted studies evaluated in White Paper.

Study First Author	EPA Study Number or Open Literature Citation	Species	Study Type
Freeman and Rayburn 2005	Environmental Toxicology and Chemistry 24(7) 1648 - 1653	*Xenopus laevis*	laboratory
Hayes *et al*. 2006a	Environmental Health Perspectives 114 (Supplement 1): 134 - 141	*Xenopus laevis*	laboratory
Hayes *et al*. 2006b	Environmental Health Perspectives 114 (Supplement 1): 40 - 50	*Rana pipiens* and *Xenopus laevis*	laboratory
Hecker *et al*. 2005a	Toxicological Sciences 86(2): 273 – 280.	*Xenopus laevis*	laboratory
Hecker *et al*. 2005b	Aquatic Toxicology 72: 383 - 396	*Xenopus laevis*	laboratory
Orton *et al.* 2006	Environmental Toxicology and Chemistry 25(1): 65 - 71	*Rana pipiens*	laboratory
Sullivan and Spence 2003.	Environmental Toxicology and Chemistry 22(3): 627 – 635.	*Xenopus laevis*	laboratory
Coady *et al.* 2004	Journal of Toxicology and Environmental Health 67 (Part A): 941 – 957. MRID 469656-05	*Rana clamitans*	laboratory
Coady *et al.* 2005	Ecotoxicology and Environmental Safety 62: 160 – 173 MRID 469656-02	*Xenopus laevis*	laboratory
Hosmer *et al.* 2007	MRID 471535-01	*Xenopus laevis*	laboratory
Murphy *et al*. 2005	Archives of Environmental Contamination 51(3): 467 – 477. MRID 469656-01	*Rana clamitans* *Rana catesbeiana* *Rana pipiens*	field
Murphy *et al*. 2006a	Aquatic Toxicology 76: 230 - 245	*Rana clamitans*	field
Murphy *et al.* 2006b	Aquatic Toxicology 77: 153 - 166	*Rana clamitans*	field
Jooste *et al*. 2005	Environmental Science and Technology 39: 5255 – 5261 MRID 469656-03	*Xenopus laevis*	mesocosm
Smith *et al*. 2005	African Journal of Herpetology 54(1): 69 – 76. MRID 469656-04	*Xenopus leavis*	field
Du Preez *et al.* 2005	African Journal of Herpetology 54(1): 61 – 68.	*Xenopus laevis*	field
Hecker *et al*. 2004.	Environmental Toxicology and Chemistry 23(8): 1996 – 2007.	*Xenopus laevis*	field
Smith *et al.* 2007	MRID 472045-01	*Rana catesbeiana*	field
Gross 2007	MRID 472044-01	*Bufo marinus*	field

CHAPTER 1

INTRODUCTION

Background

In April 2002, the Office of Pesticide Programs (OPP) of the U. S. Environmental Protection Agency (EPA) issued a science chapter that characterized the environmental fate and ecological effects of atrazine in support of the interim reregistration eligibility decision (IRED) for this chemical (IRED Science Chapter 2002). At approximately the same time that the Agency released its ecological risk assessment, new information regarding the potential effects of atrazine on amphibian development was published in the open literature (Hayes *et al*. 2002*a*). Because these new studies were published close to the time that the Agency was required to issue its regulatory decision (IRED) under a consent decree (*Natural Resources Defense Council v. Whitman*), EPA sought judicial approval to extend the deadline for issuing its atrazine IRED. In August 2002, the court agreed to an amended consent decree between EPA and the Natural Resources Defense Council (NRDC) that required EPA to issue an IRED for atrazine by January 31, 2003 and a revised IRED for atrazine by October 31, 2003. For the revised IRED, EPA agreed to consider studies conducted prior to February 28, 2003 on the effects of atrazine on amphibians. The Agency also agreed to seek external peer review of its evaluation of these studies from the Federal Insecticide, Fungicide and Rodenticide Act (FIFRA) Scientific Advisory Panel (SAP). In response to the consent decree, EPA reviewed the available literature and registrant-submitted studies on the effects of atrazine to amphibians. Because these studies were mostly related to gonadal developmental effects, EPA developed a White Paper (USEPA 2003) that focused primarily on amphibian gonadal development effects. This White Paper also discussed the nature of remaining uncertainties in evaluating the potential effects of atrazine on amphibian development and outlined the nature of future studies that could address these uncertainties.

In its 2003 White Paper, EPA concluded that overall, the lines-of-evidence based on the studies available at that time, did not show that atrazine produces consistent, reproducible effects within the range of atrazine exposure concentrations and amphibian species tested. Collectively, the available studies had deficiencies and uncertainties that limited their usefulness in interpreting potential atrazine effects. Specifically, the demasculinizing effects (decreased laryngeal dilator muscle area) could not be replicated in othermultiple laboratories. Additionally, feminizing effects (intersex/hermaphroditism/ovotestes) were observed in three different laboratory studies

whose experimental designs and results could not be easily reconciled, and these three studies reported significant effects at different concentrations: one at 25 ug/L and the other two at 0.1 ug atrazine/L. While the feminizing effects observed in these different studies were consistent qualitatively, there was no consistency across the studies in the reported dose-response relationships. Based on this inconsistency and the multiple design problems and uncertainties of the studies, a reliable determination of causality or the nature of any dose-response relationship could not be made.

In response to the uncertainties identified in the available data, the Agency identified additional information necessary to evaluate the potential causal relationship between atrazine exposure and gonadal development in amphibians. The 2003 White Paper identified additional testing tiers that could be considered to establish whether or not atrazine could cause effects on amphibian gonadal development, the nature of any associated dose-response relationship, the ecological relevancy of the effects, the plausibility of an underlying mechanism(s) of response, and the degree of interspecies variability for any effects, should a causal relationship be identified in initial studies.

EPA proposed that the studies be conducted in a tiered approach to ensure that the potential effects of atrazine are evaluated in a systematic and efficient manner, thus minimizing the level of effort and resources required, while maximizing reductions in the existing uncertainties. The first objective in this testing approach was to determine the effects of atrazine on gonadal developmental endpoints at the organism level (ovotestes, sex ratios), using high quality aquatic toxicology methods with African clawed frogs (*Xenopus laevis*). These studies were proposed to adhere to American Society for Testing Materials (ASTM) recommended standards for tadpole loading rates, husbandry, and water quality parameters. The studies were to address inconsistencies in previous research regarding the causal relationship between atrazine exposure and developmental effects to provide sufficiently robust data to determine the nature of any dose-response relationship.

EPA recommended that if the results from the initial studies were negative, then there would be no rationale to conduct further investigations. However, if the results were positive, then EPA proposed that additional studies could be designed to investigate plausible mechanisms involved in the etiology of the response since establishing a mechanistic rationale is a critical basis for inter-species extrapolation. Mechanistic understanding could also permit the

development of bioindicators that could be applied to future field studies, if deemed necessary, and would reduce the uncertainties associated with developing a causal relationship between effects on measurement endpoints and atrazine exposure.

From June 17 – 20, 2003, EPA presented its analysis, conclusions and recommendations to the FIFRA Scientific Advisory Panel, a group of independent expert scientists. In its presentation, EPA recommended additional testing initially based on laboratory studies intended to address critical design and protocol issues. The studies would be designed to provide data for adequately assessing whether atrazine exposure results in gonadal developmental effects in frogs, and if so, to determine the strength of a cause-effect relationship(s) and the nature of the underlying dose-response relationship(s) (USEPA 2003). After reviewing EPA's analysis and recommendations, the SAP concluded (SAP, 2003) that the Agency's review was thorough, the approaches and criteria were appropriate, and the conclusions were valid, given the data reviewed. The SAP also agreed that additional studies were warranted, and they concurred with the study approach described in the White Paper for addressing uncertainties identified in the available studies. There was consensus (SAP 2003) that initially laboratory studies should be conducted to confirm whether gonadal deformities occur with exposure to atrazine and that these studies should bracket concentrations at which effects were observed in previous studies.

In response to the uncertainties identified in the White Paper and based on the recommendations made by the SAP, EPA issued a Data Call-in Notice (DCI) on November 12, 2004 to Syngenta Crop Protection, Inc. (Syngenta) and other atrazine registrants. The DCI required the registrants to undertake an amphibian study, consistent with the first tier of testing described in the 2003 White Paper (USEPA 2003), to determine if exposure to atrazine can affect amphibian gonadal development. Secondary objectives of these studies were to provide information on whether or not previous observations could be replicated, to develop a sound dose-response relationship, and to determine the developmental sensitivity of the amphibian species being tested. The DCI requested that Syngenta provide draft testing protocols to the Agency for review.

In April 2005, Syngenta provided the Agency with the signed finalized version of the study protocol entitled "*Response of Larval Xenopus laevis to Atrazine Exposure: Assessment of Metamorphosis and Gonadal Morphology.*" Throughout the protocol development and implementation, the registrant kept the Agency informed and was responsive to concerns and questions expressed by the Agency. Additionally, during the in-life portion of the studies,

representatives of OPP in conjunction with personnel from EPA's Office of Enforcement Compliance and Assurance (OECA) conducted on-site inspections of each of the study laboratories, *i.e.*, Wildlife International Ltd. (Easton, MD) and the Leibniz-Institute of Freshwater Ecology and Inland Fisheries (Berlin, Germany). Following the in-life portion of the studies, OPP, OECA, and EPA's Office of Research and Development (ORD) inspected the laboratory responsible for histological analyses of samples collected during the in-life portion of the studies, *i.e.*, Experimental Pathology Labs (EPL; Sterling, VA). All laboratory inspections were conducted to ensure that the studies were proceeding according to the study protocol and its associated standard operating procedures. Additionally, raw data were reviewed along with the quality assurance procedures for each of the laboratories. During the inspection of EPL, the lead pathologist was requested to reread slide sections to determine the extent to which he could reproduce his initial determinations.

The registrant completed the required studies in 2006. In March 2007, Syngenta provided the Agency with electronic data sets for each of the amphibian studies. In June 2007, Syngenta submitted the final study report entitled "*Response of Larval Xenopus laevis to Atrazine Exposure: Assessment of Metamorphosis and Gonadal Morphology*" (Hosmer *et al*. 2007).

As indicated in the 2003 SAP meeting, EPA also agreed to review new studies of the potential effects of atrazine on amphibian gonadal development reported in the open literature. On April 4, 2007, the Agency published a Federal Register Notice (EPA-HQ-OPP-2007-0204; FR Vol 72. No. 64, pp 16357 - 16359), soliciting input on a preliminary list of relevant open literature studies that were published since the 2003 SAP White Paper. A second White Paper evaluating these new studies was developed and will be reviewed by the FIFRA SAP on October 9-12, 2007. **Table 1** summarizes all of the open literature and registrant-submitted studies reviewed in this White Paper.

Summary of Pesticide Effects Characterizations

The 2003 White Paper provided a brief overview of the process used by EPA for evaluating information used in characterizing potential risks associated with the use of pesticides. This process is more thoroughly described in the document entitled "*Overview of the Ecological Risk Assessment Process Used by the Office of Pesticide Programs*" (http://www.epa.gov/oppfead1/endanger/consultation/ecorisk-overview.pdf). In general, the risk assessment process relies on a suite of toxicity studies performed on a limited number of surrogate organisms that are intended to be representative of birds, reptiles, terrestrial-phase amphibians, mammals, fish, aquatic-phase amphibians, and aquatic invertebrates. Within these broad taxonomic groups, one acute and one chronic endpoint (usually mortality and frank measures of growth and reproduction, respectively) are selected from the available test data. Data from the most sensitive species tested within that taxonomic group are selected for estimating risk. If additional toxicity data for more species of organisms other than a surrogate species are available, the selection can be expanded to include other test species and data that meet the Agency's data quality requirements. Regardless of the extent of data available beyond the required set of toxicity studies, the risk assessment typically relies on the selection of endpoints from the most sensitive species tested in acceptable studies.

While the above mentioned surrogates and toxicity endpoints are routinely used in Agency risk assessments, they do not represent a limitation on the types of toxic endpoints that may be considered in the risk assessment. Through the evaluation of available effects data, EPA risk assessment teams may identify other effects information that provides insight on endpoints and organisms not routinely considered. Professional judgment is used by the individual chemical risk assessment team to determine whether and how available data on other toxicological endpoints are included in the risk assessment. This evaluation includes reference to data quality objectives for specific types of studies, the degree to which adequate documentation is available to evaluate the technical merit of the data, and whether the data are applicable to the assessment endpoints established for the risk assessment. In deciding whether data are applicable to assessment endpoints, the risk assessment team uses professional judgment and available lines-of-evidence to determine if the toxicological endpoints can be linked to assessment endpoints in a reasonable and plausible manner.

Consistent with the previous discussion on endpoint selection, the selection of amphibian species as the ecological entity for a risk assessment endpoint may be appropriate based on a

variety of considerations, such as a pesticide's mode of action, its use patterns, and the species habitat requirements. Because amphibians undergo profound changes during metamorphosis and occupy a variety of ecological niches during their life-span, specific species, or groups of species, may be relevant entities for formulating risk assessment endpoints for certain pesticides. Amphibians exhibit a life cycle in which conditions must be favorable for their survival in aquatic ecosystems where they breed and larvae develop, and in terrestrial habitats, where many adult amphibians reside. Living first as gilled- and skin-breathing aquatic larvae and later as completely or partly land-dwelling lung- and skin-breathing adults, these animals rely on a wide range of mechanisms for interfacing with their environments (Rowe *et al*. 2003). Unlike many organisms that have either keratinized and/or scaled outer surfaces, the amphibian skin is thin and is used as a respiratory membrane for gas exchange and water absorption (Henry 2000; Stebbins and Cohen 1995). During the larval stage, many amphibians rely on filter feeding although, as a group, they are considered the primary vertebrate predator (Duellman and Trueb 1986) for invertebrates in many freshwater and moist terrestrial environments.

DOCUMENT OBJECTIVE

The intent of this 2007 White Paper is to critically review the available literature and registrant-sponsored studies that were conducted subsequent to the 2003 SAP. This White Paper focuses on a review of relevant studies that examine the effects of atrazine alone on amphibian gonadal development and evaluates whether there is sufficient information to conclude that atrazine exposure does or does not result in toxic effects on amphibian gonadal development. In addition to determining whether atrazine expsoure results in adverse effects on amphibian gonadal development, this paper examines whether such a causal relationship adheres to a consistent concentration-response relationship. In doing so, the Agency examines the appropriateness of the experimental design and any major and/or minor uncertainties in the study methods that could confound efforts to interpret the study results.

The structure of this 2007 White Paper is similar to the 2003 White Paper (USEPA 2003) and includes an overview of the regulatory history of atrazine in Chapter 1. Chapter 2 discusses the results of the studies conducted in response to the DCI. In Chapter 3, open literature study reviews are grouped into laboratory and field investigations each noting the study methods and results, followed by a discussion of specific issues associated with each study. Following these reviews, Chapter 4 discusses strengths and limitations found in the laboratory and field studies

relative to recommendations made by EPA (USEPA 2003), with concurrence from the 2003 SAP (SAP 2003), to ascertain the usefulness of studies in characterizing the potential developmental effects of atrazine on amphibians for the purposes of risk assessment. Chapter 5 presents the conclusions of the White Paper. Finally in Chapter 6, EPA poses a series of questions that require input from the SAP on the Agency's study evaluations and its conclusions related to establishing a causal relationship between atrazine exposure and effects on amphibian development.

CHAPTER 2

STUDIES IN RESPONSE TO DATA CALL-IN

In 2003, the FIFRA Scientific Advisory Panel (SAP) issued a report (SAP 2003) regarding the potential developmental effects of atrazine on amphibians. In this report, the SAP reviewed EPA's interpretation of seventeen studies and responded to questions posed to the SAP on June 17 - 20, 2003. These questions were summarized in the 2003 White Paper (USEPA 2003) in which EFED, in conjunction with the Mid-continent Ecology Division (MED) of the ORD/EPA, summarized seventeen studies consisting of both open literature and registrant-submitted laboratory and field studies involving both native and non-native species of frogs. In general, the SAP agreed (SAP 2003) with the Agency's assessment of the available studies on the effects of atrazine on amphibian gonadal development. The SAP also agreed with the proposed multi-tiered study process for addressing uncertainties regarding the effects of atrazine on amphibian gonadal development. While both the Agency and the SAP agreed that there were sufficient data to formulate a hypothesis that atrazine may impact amphibian gonadal development, there were insufficient data to refute or confirm the hypothesis at that time. In response to the SAP's report (SAP 2003), the Agency reviewed the registrant's detailed protocols for Tier I-level testing consistent with the process identified in the 2003 White Paper (USEPA 2003). In reviewing the registrant's study design elements, the Agency considered additional reference materials identified by the SAP.

While the SAP recommended both laboratory and field studies as means of determining effects, the Panel concurred with the Agency that laboratory studies are an appropriate and critical means of determining whether atrazine exposure causes amphibian gonadal developmental effects (SAP 2003). The Agency agreed that field studies are necessary to determine the ecological relevancy of the purported gonadal deformities; however, it was important first to establish that a causal relationship exists and that it adheres to some consistent concentration-response relationship before its relevancy should be evaluated. Thus, initial studies focused on establishing whether a causal relationship exists and the nature of the concentration-response relationship should one exist. Although the SAP recommended that a native species would be more suitable for confirmatory testing, there were no data available to the Panel's knowledge indicating significant differences between developmental pathways in the non-native

Xenopus and native frogs such as *Rana* (SAP 2003). Therefore, initial studies focused on *Xenopus* where a considerable body of information already exists.

In response to the Agency's 2003 White Paper and concurrence by the FIFRA SAP and in anticipation of a data call-in associated with the interim reregistration eligibility decision, the technical registrant, Syngenta Crop Protection, submitted a document entitled "*Design Elements for the Study Protocol: Response of Larval Xenopus laevis to Atrazine Exposure: Assessment of Metamorphosis and Gonadal Morphology*" in August 2003 (Hosmer 2003). The document focused on examining the potential effects of atrazine on gonadal morphology in developing *X. laevis*. The Agency provided a response (USEPA 2003b) to the draft proposal indicating that while the proposal lacked sufficient details to completely define the conduct of Tier I testing, it did provide a reasonable outline of the general study approach. Additionally, the Agency provided recommendations to better enable the registrant to complete the necessary design elements. On November 12, 2004, the Agency issued a Data Call-in (DCI) Notice to Syngenta and other atrazine registrants, and in April 2005, Syngenta provided the Agency with the signed finalized version of the study protocol entitled "*Response of Larval Xenopus laevis to Atrazine Exposure: Assessment of Metamorphosis and Gonadal Morphology*" (Hosmer 2005) The protocol reflected several years of preliminary tests and pilot studies to establish suitable husbandry and exposure conditions to reduce the confounding effects identified in the Agency's 2003 White Paper (USEPA 2003) regarding previous studies.

As stated in the 2003 White Paper (USEPA 2003), the tiered approach for conducting studies was partitioned into five distinct phases following a reductionist approach (**Figure 1**). The objective of the first phase was to conduct studies that determine whether or not atrazine exposure causes changes in apical effects (*e.g*., hermaphroditism) related to gonadal development and reproduction. A secondary objective of this phase was to provide information on the repeatability of previous observations, develop a dose-response relationship, and determine the developmental sensitivity of the test species, *X. laevis*.

Although not stipulated by the DCI, the registrant decided to conduct two separate (Tier 1) studies to examine the reproducibility of the results (Hosmer *et al*. 2007). The in-life portion of the studies was conducted at Wildlife International, Ltd. (WLI) in Easton, Maryland, and at the Leibniz Institute of Freshwater Biology and Inland Fisheries (IGB) in Berlin, Germany. Both testing facilities used the same study protocol and had relatively similar standard operating

procedures (SOPs) for their study-related laboratory equipment/procedures. Both laboratories were required to conduct their studies following Good Laboratory Practice (GLP) standards as stipulated under FIFRA (40 CFR Part 160) and were subject to both internal and external quality assurance (QA) reviews. All histology samples were processed by Experimental Pathology Laboratories (EPL) in Sterling, VA, and all statistical analyses were conducted by Sielken and Associates (Bryan, TX). Similar to the in-life portion of the study, all of the histological analyses were subject to GLP and QA inspections. While statistical analyses were not GLP compliant, their methodologies and associated assumptions were reviewed by EPA prior to use.

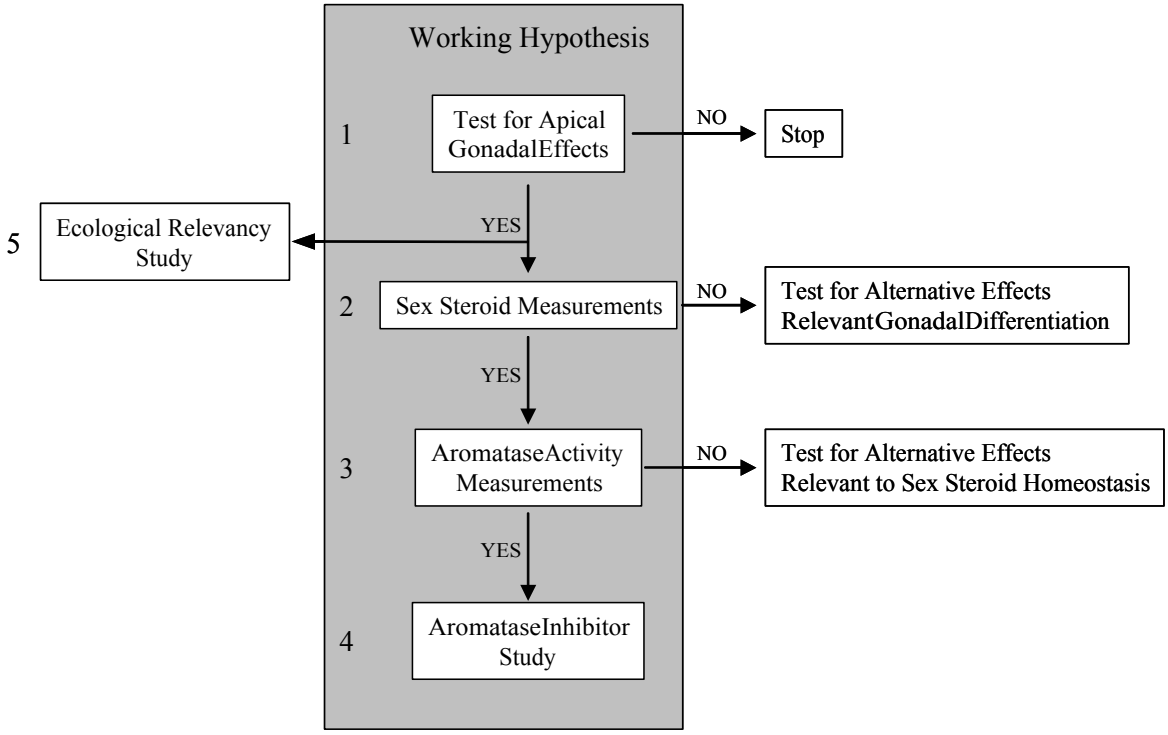

Figure 1. Scheme for laboratory studies to determine the effects of atrazine on gonadal differentiation in anuran amphibians. The grey block contains the working hypothesis for atrazine action on gonadal differentiation, which is partitioned into four logical phases. See text for details.

Description and Results of Study

According to the study authors (Hosmer *et al*. 2007), the objective of the Phase (or Tier) I studies was to examine the potential effects of atrazine exposure on sexual differentiation and gonadal development in the African clawed frog as indicated by measures of sex ratio, mixed sex animals (both gender tissues within a single gonad), intersex animals (left/right gonads of different gender), and frequency of testicular oocytes. Secondary or supplemental gross and histological observations and measures, related primarily to gonad size, position, and appearance or structure, were also included to identify other potential atrazine-related findings.

African clawed frogs were obtained from a commercial supplier (Xenopus I, Dexter, Michigan) using 10 different mating pairs for each study. The treatment design included a positive control using 17β-estradiol (97%; Sigma-Aldrich) and five concentrations of atrazine (97.1%; Syngenta) at 0.01, 0.1, 1, 25, and 100 µg active ingredient (a.i.)/L. These test solutions

were each delivered via a continuous flow-through system to individual 9-L glass aquaria each containing 7 L of exposure solution. The flow rate through each of the tanks, which was 50 L/tank/day, was equivalent to a turnover rate of greater than 7 tank volumes each day and was sufficient to maintain a tadpole loading rate equivalent to 1 g/L/day. Dilution water for IGB was obtained from municipal city supply, while WLI relied on filtered well water. Beginning on Day 28 of exposure, tanks were aerated.

A randomized nested design was used for the experiment. Sixty-four aquaria (tanks) were randomly divided into 16 groups of 4 tanks each (clusters). These clusters were then randomly assigned to one of 16 flow systems (two 8-channel peristaltic pumps) that administered treatment concentrations to the tanks during the study. Each of the atrazine treatment groups and the positive control group were randomly assigned to 2 pumps (4 for the negative control) for a total of 8 tanks per treatment group (16 for the negative control). Each tank was randomly assigned 25 African clawed frog larvae, for a total of 25x8=200 subsamples (larvae) per treatment and positive control group (400 per negative control.) Unless statistically significant cluster effects could be shown, each of the eight tanks per treatment group was considered an independent experimental unit for this study.

The nominal concentration of the positive estradiol control (0.2 µg/L) was selected based on preliminary studies showing that this exposure concentration resulted in a 50% feminization of male frogs (EC_{50}). Atrazine concentrations were selected to bracket concentrations reported in previous studies, *i.e.*, 0.1 – 25 µg a.i./L, to result in effects on amphibian gonadal development. Exposure was initiated on Day 8 post-fertilization (NF Stage 46 – 48) and continued until metamorphosis was completed (NF Stage 66) or after 75 days, whichever came first. Exposure concentrations were measured every week; atrazine and estradiol concentrations were analyzed using liquid chromatography and tandem mass spectrometry (LC/MS/MS). The limit of quantification for atrazine was 0.01 µg/L. Initially, atrazine exposure concentration was measured using an enzyme-linked immunabsorbant assay (ELISA); however, the ELISA method was abandoned due to high variability in the test results compared to the LC/MS/MS (pers. comm., Alan Hosmer, Study Director, 2007). Frogs were fed daily a solution of Sera Micron® commercial feed (Sera GmbH, Heinsberg, Germany); the commercial diet was analyzed for pesticides including atrazine and for the presence of phytoestrogens.

Environmental conditions (temperature, light intensity, dissolved oxygen, pH, water hardness, alkalinity, specific conductance, nitrate, and ammonia) were measured throughout the study. Tanks were cleaned daily. Tank water temperatures were maintained either through the use of a walk-in environmental chamber or through a water bath.

At completion of metamorphosis or at exposure termination on Day 75, frogs were euthanized using methyl tricaine sulfonate, snout-vent length and body weight were measured, and gross morphology (with the aid of a stereomicroscope) of the gonads and kidneys were recorded. Animals were subsequently fixed in Bouin's solution for histological analysis. Any larvae that were found dead prior to NF Stage 58 were not considered for gross gonadal assessments because of their immaturity.

According to the study results (Hosmer *et al.* 2007), the mean concentration of atrazine in the three highest treatments (1.0, 25 and 100 µg/L) ranged from 74 – 99% of nominal across what was termed the "critical window for feminization" (NF Stage 42 – 54). Measured concentrations in the 0.01 and 0.1 µg/L treatments ranged from 52 – 91% of nominal through the "critical window." Across the entire study period and atrazine treatments, average measured concentrations ranged from 56 – 101% of nominal. For the positive controls, average measured concentrations during the "critical window" ranged between 62 – 66% and over the entire period ranged between 81 – 84% of nominal.

In the WLI study, an entire tank was terminated after significant mortality in the 1.0 µg/L atrazine treatment due to a "microbial bloom" during Days 16 and 17 of the study; WLI removed this tank from the data before they submitted it for analysis. A cluster of tanks in the negative control group were dropped from the study due to atrazine contamination; a second cluster of negative controls were dropped due to a microbial bloom. The elimination of these two negative control clusters reduced the negative control sample size from 16 to 8 tanks. In addition there were 58 tadpoles that died across all treatments between stage 46 and 66, of which 51 had no necropsy performed, and therefore were not included in the dataset to be analyzed. After accounting for two animals that failed to complete metamorphosis, statistical analysis for WLI was based on a dataset containing 1312 valid subsamples (*i.e.* frogs) out of the initial 1600 that were planned. For IGB this number totaled 1555. Most analyses then were carried out separately for the two sexes on datasets roughly half the sizes of the above.

There were four datasets that were statistically analyzed: gross morphology data for WLI, histology data for WLI (performed by EPL), gross moprhology data for IGB, and histology data for IGB (performed by EPL). The variables that were measured and analyzed are listed below.

Gross Morphology Measurements:

Mean values calculated for each treatment group

- Age at completion of metamorphosis (days)
- Body weight (g) at metamorphosis
- Snout-to-vent length (mm) at metamorphosis
- Gonadal image area (mm^2)

Mean percent values calculated for each treatment group in the gross morphology analysis:

- Tadpole mortality
- Frequency of males
- Frequency of intersex
- Frequency of mixed sex
- Mixed tissue types
- Failure to complete metamorphosis
- Gonadal adhesions
- Gonadal aplasia
- Gonadal bifurcation
- Gonadal pearling
- Internal melanophores in testes
- Gonadal hypertrophy
- Gonadal displacement
- Gonadal hypoplasia
- Gonadal segmental translucence
- Fused gonads
- Gonadal segmental hypertrophy
- Gonadal segmental hypoplasia
- Gonadal deformity
- Gonadal segmental aplasia
- Gonadal translucence
- Melanophores decreased in ovaries

- **Histology Measurements:**
- Segmental hypoplasia
- Fused kidneys
- Segmental aplasia
- Mineralization
- Decreased germ cells in gonad
- Other renal diagnoses

- Males only
  - Dilated testis tubules
  - Dividing gonadocytes in testes
  - Internal melanophores in testis

- Females only
  - One or more immature ovaries
  - Decreased ovarian pigmentation
  - Decreased ovarian cavity size
  - Increased ovarian cavity size
  - Ovarian interstitial fibrosis
  - Mononuclear cell infiltrates in ovary
  - One or more unable to stage ovaries

In cases where the gross examination identified animals as mixed sex but the histological analysis did not, the animal was identified as mixed sex. In instances where the gross analysis was able to positively identify a sex (<1%) but the histological analysis was unable to, then the animal was identified according to the gross determination. Aside from these two scenarios, the sex variable for the gross statistical analysis was identified according to the histological determination. If neither gross nor histological methods could identify a sex, then the histological data label for "unidentified" was retained in the data set. This occurred for 7 animals at WLI and 4 animals at IGB.

Animals that died (58 at WLI; 43 at IGB) or failed to metamorphose (2 and 1, respectively), were assigned a sex of "missing." Both males and females were each analyzed for all response variables with the exception of a few that are specific to either ovaries or testes. Mixed sex and intersex animals were only included in variables testing for mixed sex tissue types and gonadal intersex. Based on gross inspection, however, no cases of either were seen in the atrazine treated animals at either lab.

Decision-based statistical hypothesis tests were conducted on the study data to test whether (stated generally) African clawed frogs exposed to atrazine exhibited gonadal developmental effects measures that were statistically significantly different compared to African clawed frogs not exposed to atrazine. The continuous response variables, which included age at completion of metamorphosis, body weight, and snout-to-vent length, were analyzed using parametric statistical tests: one-way ANOVA F-test, independent Student's t-tests, pair-wise

ANOVA contrast comparisons. The remaining response variables (*e.g*., tadpole mortality, presence of mixed sex/intersex animals, presence of gonadal abnormalities), most of which were categorical response variables, were analyzed using non-parametric tests. The non-parametric Jonckheere-Terpstra test was conducted on all response variables to test whether a monotonic trend exists in the data. In nearly all cases, based upon *a priori* hypotheses, EPA tested for a one-sided increasing effect, except for the frequency of males, which was tested as a one-sided decreasing effect. For the variables, age at completion of metamorphosis, body weight, snout-to-vent length, and gonadal image area, where no hypothesized directional outcomes were made, two-sided tests were conducted. [Note that unlike the Agency, the study authors conducted two-sided, rather than one-sided tests for tadpole mortality, failure to complete metamorphosis, and frequency of males.] The two-sided tests were conducted at the 10% significance level (*i.e*., $p<0.10$), while one-sided comparisons were considered to be significant when $p<0.05$. Probability values within this range, however, are specifically reported below.

Results for normally-distributed continuous response variables with equal variances are reported with the mean and standard deviation as the measures of central tendency and variability, along with 95% confidence intervals about the mean; all others report the median and range of minimum and maximum values. Binary and ordinal frequency values are expressed both in terms of the mean and the median of the percent mortality in each of the eight tanks. A full set of summary data is contained in **Appendix 2**.

Positive Control

The positive estradiol (0.2 µg/L) control group showed significance for the following variables: age at completion of metamorphosis for males and females, gonadal image area in males, mixed tissues types, frequency of males among all animals, and frequency of mixed sex, as well as for gonadal segmental aplasia and gonadal translucence for both males and females.

Cluster effects within treatments

For each of the response variables, no statistically significant differences were detected between the two clusters of tanks within each of the treatment groups, with the exception being dilated testicular tubules (Grade >0) in the control group of males at IGB. It should be noted, however, that small numbers for the test afforded limited power for detecting cluster effects in the study.

Effects of Atrazine

Based on the study results, neither mortality nor time to metamorphosis was affected by atrazine treatment for either males or females. Mortality for tadpoles is presented for both study labs in **Table 2**. Tanks that were dropped from the study or individual tadpole deaths, which were classified as mishaps, are not included in the calculations.

Table 2. Summary of percent mortality (mean and range) by treatment group and study laboratory.

Testing Facility	Negative Control	Positive E2 Control	Atrazine µg/L				
			0.01	0.1	1.0	25	100
WIL	6.5 (0-20)	2.5 (0-8)	1.5 (0-4)	5.0 (0-12)	4.6 (0-12)	6.0 (4-12)	3.5 (0-8)
IGB	2.5 (0-8)	3.0 (0-8)	4.0 (0-16)	2.0 (0-8)	2.5 (0-8)	2.5 (0-4)	3.0 (0-12)

Average time to metamorphosis ranged from 50.9 to 52.9 days; there was no statistical difference in time to metamorphosis for the atrazine treated tanks (**Table 3**).

Table 3. Mean age in days post-hatch and (standard error) at completion of metamorphosis for male and female frogs across treatment groups and study laboratories.

Study Laboratory	Gender	Negative Control	Positive E2 Control	Atrazine µg/L				
				0.01	0.1	1.0	25	100
WIL	Male	52.2 (.62)	55.2*(.82)	51.6 (.33)	52.6 (.79)	52.9 (.90)	51.4 (.31)	52.1 (.56)
	Female	52.9 (.39)	54.0 (.49)	51.6 (.51)	52.0 (.42)	51.9 (.52)	52.7 (.36)	52.9 (.55)
IGB	Male	50.9 (.50)	54.8* (.58)	51.5 (.75)	51.4 (.82)	51.5 (.89)	50.5 (.59)	51.1 (.49)
	Female	51.7 (.45)	53.4* (.69)	50.4 (.60)	51.9 (.37)	50.9 (.48)	51.8 (.41)	51.6 (.58)

*statistically significant estradiol p=.013(WIL); p=.0001(IGB-M); p=.043(IGB-F)

Sex ratio, based on phenotypic sex and expressed as the percentage of males, was not significantly different for atrazine-treated versus negative control groups for either laboratory (**Table 4).**

Table 4. Mean percentage (and range) of males, based on phenotypic sex.**

Study Laboratory	Negative Control	Positive E2 Control	Atrazine µg/L				
			0.01	0.1	1.0	25	100
WIL	49 (27-58)	20* (8-37)	54 (32-71)	46 (43-54)	44 (26-60)	49 (35-64)	46 (28-68)
IGB	47 (28-71)	27* (8-38)	47 (30-67)	45 (33-61)	50 (36-63)	48 (42-63)	52 (29-67)

*Statistically significant estradiol p=.0003 (WIL); p=.0006(IGB)

** Mixed sex animals were not included.

For males, there was no significant difference in weight or snout-to-vent length (SVL) at metamorphosis between the negative control and atrazine-treated animals; average male weight ranged between 0.51 – 0.58 g (**Table 5**). For females, 7% reductions in body weight (g) at metamorphosis were detected as significantly less than controls at IGB for animals treated with 0.01, 1 and 100 µg/L atrazine; however, there was no statistical difference in weight for animals treated with 0.1 and 25 µg/L. These data suggest the absence of a monotonic dose response. Female body weight at metamorphosis was not significantly different for animals in the WLI study.

Table 5. Summary of mean (standard error) body weights (g) at metamorphosis for male and female frogs by treatment and study laboratory.

Study Laboratory	Gender	Negative Control	Positive Control	Atrazine µg/L				
				0.01	0.1	1.0	25	100
WIL	Male	0.55 (.01)	0.59 (.02)	0.58(02)	0.56(.02)	0.56(.02)	0.57(.01)	0.58(.02)
	Female	0.61 (.01)	0.62(.01)	0.58(.02)	0.57(.02)	0.58(.02)	0.60(.01)	0.58(.02)
IGB	Male	0.54 (.01)	0.57(.02)	0.52(.01)	0.52(.01)	0.51(.02)	0.52(.01)	0.52(.02)
	Female	0.56 (.01)	0.56(.01)	0.52(.01)*	0.54(.01)	0.52(.01)*	0.56(.01)	0.52(.01)*

*significantly different (p=0.0187) from negative controls; p<.015 in pairwise

Mean length at metamorphosis for males ranged between 17.8 – 18.6 mm; female mean length at metamorphosis ranged between 17.8 – 18.8 mm (**Table 6**). Female SVL at metamorphosis was significantly less than controls at IGB for animals treated with 0.01, 1, and 100 µg/L atrazine; however, there was no statistical difference in weight for animals treated with 0.1 and 25 µg atrazine/L. Similar to body weights, there was no clear pattern in the reduction in length with increasing atrazine concentration; the percentage reductions in length at 0.1, 1.0, and 100 µg/L were 2.7%, 2.2%, and 3.3%, respectively. Also, similar to what was observed for body weight, the significant effects on length measured at IGB were not observed at WLI. At WLI, atrazine-treated animal length at metamorphosis was not statistically different from control animals.

Table 6. Summary of mean (standard error) snout-to-vent length (mm) at metamorphosis for male and females frogs by treatment and study laboratory.

Study Lab	Gender	Negative Control	Positive E2 Control	Atrazine µg/L				
				0.01	0.1	1.0	25	100
WIL	Male	18.3 (.16)	18.6 (.20)	18.4 (.15)	18.3 (.18)	18.3 (.20)	18.4 (.14)	18.5 (.18)
	Female	18.7 (.14)	18.8 (.09)	18.5 (.11)	18.4 (.17)	18.4 (.21)	18.6 (.12)	18.4 (.18)
IGB	Male	18.1 (.09)	18.4 (.27)	18.0 (.08)	18.0 (.17)	17.8 (.12)	17.8 (.12)	18.0 (.17)
	Female	18.4 (.12)	18.6 (.14)	17.9*(.08)	18.2 (.11)	18.0* (.10)	18.3 (.07)	17.8* (.19)

*significantly different from negative controls; p=.009(.01ug/L); p=.021(1ug/L); p=.004(100ug/L)

At the gross morphological level, a number of response variables showed very low counts across most of the treatment and negative control groups. At IGB, with the exception of gonadal hypertrophy and gonadal hypoplasia, which ranged up to 37% and 49% in some treatments, the remaining gross morphological variables reported "0" in at least 89% of the observations. No observations were recorded among atrazine-treated groups for mixed-tissue types, mixed sex, or intersex based upon gross observation, gonadal adhesions, gonadal aplasia, bifurcation, pearling, and gonadal deformity (males). One animal at the 25 ug/L level failed to complete metamorphosis, and one at the same level showed internal melanophores in testes. At WLI there were no observations of mixed sex or intersex among atrazine groups at the gross morphological level, but for all other variables at least one or two occurrences were reported for one or more atrazine treatment groups.

For most response variables particularly secondary response variables, analyses were conducted separately for males and females so that percent values were calculated out of approximately 12 to 13 animals per tank when the ratio of males to females was even. An effect whose occurrence is less frequent than 8-10% may not have a high likelihood of being seen without higher sample sizes per tank. These, presumably, would have been difficult to achieve, however, both logistically and for reasons of husbandry.

Certain gross morphological effects as well as histologically diagnosed effects were reported more commonly at both labs, and are presented below. (For histological effects, all results are reported for severity > grade 0, which includes all severity levels for a particular lesion). The most frequently observed histological lesions are depicted in **Appendix 3**.

Gonadal hypoplasia was significant at 0.1 µg/L for males at WLI, similar to results for

the positive control (**Table 7**). For gonadal segmental hypoplasia, results were significant for IGB females at 0.1 and 25 ug/L, similar to the estradiol responses (**Table 8**).

Table 7. Summary of percent gonadal hypoplasia >0 (and ranges) by treatment group and study laboratory

Testing Facility	Gender	Negative Control	Positive E2 Control	Atrazine µg/L				
				0.01	0.1	1.0	25	100
WIL	Male Female	**2** (0-8) **32** (10-55)	**12** (0-60)* **37** (21-55)	**2** (0-9) **27** (8-42)	**12** (0-20)* **34** (18-50)	**4** (0-20) **39** (30-50)	**5** (0-10) **30** (0-50)	**4** (0-13) **33** (21-63)
IGB	Male Female	**6** (0-27) **38** (20-57)	**3** (0-11) **50** (37-64)*	**9** (0-22) **49** (23-86)	**7** (0-15) **30** (7-57)	**5** (0-15) **36** (22-53)	**10** (0-30) **40** (23-69)	**6** (0-1742) **32** (11-50)

*statistically significant overall p=0.062; (p=0.006 at 0.1ug/L)
p=.019 E2 males (WIL); p=.003 E2 females (IGB)

Table 8. Summary of percent gonadal segmental hypoplasia >0 (and ranges) by treatment group and study laboratory

Testing Facility	Gender	Negative Control	Positive E2 Control	Atrazine µg/L				
				0.01	0.1	1.0	25	100
WIL	Male Female	**4** (0-15) **12** (0-20)	**9** (0-33) **15** (7-25)	**3** (0-8) **18** (0-44)	**6** (0-11) **10** (6-17)	**4** (0-11) **10** (0-17)	**5** (0-14) **16** (0-25)	**2** (0-14) **16** (0-46)
IGB	Male Female	**2** (0-12) **3** (0-22)	**0** (0) **7** (0-15)*	**0** (0) **3** (0-13)	**3** (0-11) **8** (0-19)*	**1** (0-9) **3** (0-8)	**0** (0) **8** (0-15)*	**2** (0-9) **7** (0-25)

*statistically significant overall p=0.095; (p=~.02 at 0.1 and 25 ug/L)
p=.028 E2 females

A statistically significant difference was found for gonadal segmental hypertrophy among females at IGB at the 25 µg/L level (**Table 9**). The frequency at which this effect was reported, however, was low across all groups.

Table 9. Summary of percent gonadal segmental hypertrophy >0 (and ranges) by treatment group and study laboratory

Testing Facility	Gender	Negative Control	Positive E2 Control	Atrazine µg/L				
				0.01	0.1	1.0	25	100
WIL	Male Female	**0** (0) **0** (0)	**4** (0-20) **0** (0)	**1** (0-8) **0** (0)	**0** (0) **2** (0-8)	**0** (0) **0** (0)	**2** (0-10) **0** (0)	**1** (0-7) **0** (0)
IGB	Male Female	**0** (0) **1** (0-7)	**0** (0) **0** (0)	**1** (0-7) **2** (0-7)	**0** (0) **0** (0)	**0** (0) **1** (0-9)	**0** (0) **5** (0-15)*	**1** (0-9) **1** (0-7)

*statistically significant overall p=0.091; (p=0.015 at 25 µg/L)

Gonadal segmental translucence showed significance at WLI for both males and females, and at several treatment levels and positive control for females (**Table 10**).

Table 10. Summary of percent gonadal segmental translucence >0 (and ranges) by treatment group and study laboratory

Testing Facility	Gender	Negative Control	Positive E2 Control	Atrazine µg/L				
				0.01	0.1	1.0	25	100
WIL	Male Female	**1** (0-7) **1** (0-6)	**9** (0-33) **5** (0-11)*	**3** (0-8) **4** (0-11)	**1** (0-10) **7** (0-8)*	**3** (0-11) **4** (0-13)	**0** (0) **8** (0-13)*	**9** (0-14)* **5** (0-15)*
IGB	Male Female	**8** (0-20) **1** (0-11)	**16** (0-50) **1** (0-8)	**3** (0-17) **0** (0)	**8** (0-20) **1** (0-10)	**6** (0-20) **0** (0)	**6** (0-18) **1** (0-9)	**4** (0-9) **0** (0)

*For pairwise comparisons p=0.001 for males at 100 µg/L; p~.001 for females at 0.1 and 25 µg/L; p=0.022 at 100 µg/L
p=.041 for E2 females

Mean gonadal image area showed a significant increase in males at WLI at 100 ug/L, similar to the results for the positive control (**Table 11**). Measurements at IGB, however, showed a significant downward trend.

Table 11. Summary of mean gonadal image area (and standard error) by treatment group and study laboratory.

Testing Facility	Gender	Negative Control	Positive E2 Control	Atrazine µg/L				
				0.01	0.1	1.0	25	100
WIL	Male Female	**.84** (0.02) **1.33** (0.05)	**.91** (0.02)* **1.36** (.03)	**.88** (0.01) **1.32**(.04)	**.83** (0.01) **1.27**(0.06)	**.84** (0.01) **1.32**(.04)	**.86** (0.02) **1.34**(0.03)	**.90**(0.02)* **1.31**(.06)
IGB	Male Female	**.92** (0.02) **1.68** (0.04)	**1.08** (0.07)* **1.65** (0.06)	**.90** (0.02) **1.53** (0.05)	**.86** (0.02) **1.61**(0.04)	**.85** (0.02) **1.61**(0.04)	**.87** (0.02) **1.60**(0.04)	**.84** (0.03) **1.570**(.03)

*statistically significant p=0.016 at 100 µg/L
p=.017 for E2 males (WIL); p=.007 for E2 males (IGB)

The above responses were detected as being significant overall (*i.e.* at least one treatment or control mean different from at least one other treatment or control mean), and were also shown to be significant in pairwise comparisons with the negative control. A number of other responses were significant in the overall test, but the largest difference among groups was often the difference between two atrazine treatments.

Percent of fused kidneys in males showed overall significance at WLI, and for the 1.0 µg/L comparison with negative control p=0.032 (**Table 12**). The largest differences contributing to the overall statistical test, however, appear to be between atrazine-treated groups.

Table 12. Summary of percent fused kidneys >0 (and ranges) by treatment group and study laboratory.

Test Facility	Gender	Negative Control	Positive E2 Control	Atrazine µg/L				
				0.01	0.1	1.0	25	100
WIL*	Male Female	**11** (0-31) **16** (0-33)	**4** (0-17) **17** (5-29)	**7** (0-18) **22** (0-42)	**6** (0-18) **22** (0-45)	**21** (9-33)* **19** (8-36)	**12** (0-18) **20** (0-38)	**16** (9-33) **27** (6-44)
IGB	Male Female	**10** (0-30) **14** (0-36)	**18** (0-60) **19** (13-36)	**12** (0-25) **15** (0-36)	**13** (0-20) **18** (0-50)	**14** (7-23) **16** (0-42)	**13** (0-30) **12** (0-31)	**22** (7-42) **19** (0-40)

*statistically significant overall p=0.014; (p=0.032 at 1.0ug/L)

Dilated testicular tubules showed overall significance at WLI (**Table 13**), although the largest contributing differences appear to be between atrazine-treated groups. None of the means for treated groups reached the percentage of diluted testicular tubules observed in the positive control.

Table 13. Summary of percent dilated testicular tubules >0 (and ranges) by treatment group and study laboratory.

Test Facility	Negative Control	Positive E2 Control	Atrazine µg/L				
			0.01	0.1	1.0	25	100
WIL	**11** (0-23)	**74** (0-100)*	**8** (0-25)	**7** (0-17)	**5** (0-14)	**6** (0-18)	**16** (9-27)*
IGB	**11** (0-44)	**63** (33-100)*	**11** (0-29)	**14** (0-27)	**10** (0-36)	**18** (0-33)	**11** (6-19)

*statistically significant overall p=0.039; (p=0.076 at 100ug/L);
p<.0001 for E2 males at both labs

No significant difference among treatments was found at WLI (**Table 14**) for incidence of internal testicular melanophores. While an overall difference was found at IGB, it was likely due to the difference between two atrazine-treated groups. Pair-wise comparisons with the negative control were not significant.

Table 14. Summary of percent internal testicular melanophores >0 (and ranges) by treatment group and study laboratory .

Testing Facility	Negative Control	Positive E2 Control	Atrazine µg/L				
			0.01	0.1	1.0	25	100
WIL	**81** (62-100)	**100** (all)*	**74** (55-100)	**77** (56-90)	**65** (43-83)	**66** (40-93)	**77** (63-100)
IGB*	**59** (11-82)	**78** (60-100)*	**59** (27-82)	**66** (47-89)	**58** (20-77)	**64** (40-80)	**49** (20-73)

statistically significant overall
p=.002 for E2 (WIL); p=.043 for E2 (IGB)

There was no statistical significance for decreased ovarian cavity size at WLI (**Table 15**). At IGB, the overall test for differences was not significant (p=0.122), but the test for trend showed significance (p<0.05) at the 100, 25, and 1.0 µg/L treatment levels.

Table 15. Summary of percent observed decreases in ovarian cavity size >0 (and ranges) by treatment group and study laboratory.

Testing Facility	Negative Control	Positive E2 Control	Atrazine µg/L				
			0.01	0.1	1.0	25	100
WIL	**14** (0-27)	**26** (13-43)	**7** (0-14)	**14** (0-25)	**15** (9-25)	**18** (8-27)	**12** (0-25)
IGB*	**7** (0-22)	**9** (0-19)	**14** (0-29)	**9** (0-22)	**12** (0-20)*	**18** (0-27)*	**11** (0-33)*

* significant trend test

Observations of decreased germ cells were prevalent in animals for both sexes, and higher in the males at both labs (**Table 16**). Neither lab showed significant differences between atrazine-treated groups and negative controls, however.

Table 16. Summary of percent observed decreased germ cells >0 (and ranges) by treatment group and study laboratory

Test Facility	Gender	Negative Control	Positive Control	Atrazine µg/L				
				0.01	0.1	1.0	25	100
WIL	Male Female	**81** (67-93) **50** (31-58)	**69** (33-100) **57** (37-71)	**82** (69-93) **54** (42-71)	**85** (62-100) **62** (39-92)	**84** (64-100) **54** (40-67)	**78** (55-88) **54** (29-69)	**83** (71-100) **58** (36-78)
IGB	Male Female	**70** (45-91) **42** (14-67)	**72** (50-100) **42** (31-56)	**70** (43-89) **47** (33-64)	**71** (46-89) **53** (31-78)	**72** (46-100) **43** (33-58)	**69** (50-92) **39** (23-46)	**71** (55-88) **48** (22-63)

Dividing gonadocytes (all lesions) were not significant for atrazine treated animals at either lab (**Table 17**).

Table 17. Summary of percent dividing gonadocytes in testes >0 (and ranges) by treatment group and study laboratory

Test Facility	Negative Control	Positive Control	0.01 µg/L	0.1 µg/L	1.0 µg/L	25 µg/L	100 µg/L
WIL	**9** (0-25)	**41** (0-67)*	**17** (0-38)	**22** (9-40)	**14** (7-18)	**18** (0-29)	**16** (0-29)
IGB	**16** (0-33)	**45** (0-78)*	**23** (0-58)	**16** (0-29)	**27** (10-46)	**22** (0-36)	**15** (0-25)

p<.0001 for E2 animals at both labs

There was little overlap in significant findings between WLI and IGB. As presented above, several secondary gross morphological effects showed significance at both the overall

level and in pair-wise comparisons with the negative control. At WLI, gonadal image area for males was significant at 100 µg/L, representing a 7% increase from the control mean. Estradiol positive controls showed a similar, though more pronounced, increase for this measure. Gonadal segmental translucence was statistically significant for males at 100 µg/L as well as for females at the 0.10, 25, and 100 µg/L treatments. A similar increase was seen in the positive controls, although the level of significance was $.05<p<.10$. Gonadal hypoplasia in males was found to be significant at 0.1 µg/L at WLI, consistent with the effect for estradiol. IGB did not report similar significance in findings for the atrazine-treated groups for any of these response variables. At IGB, gonadal segmental hypoplasia for females was significant at 25 and 0.1 µg/L (similar to the increase for the positive control), as was segmental hypertrophy for females at 25 µg/L. There was no evidence of a monotonic dose-response relationship for these observations.

Most of the histologically diagnosed responses presented above were detected as being significant overall, but demonstrated larger differences between two treatments than between individual treatments and control. Among all effects, responses that showed this kind of variability included gonadal displacement in males at WLI (gross morphology), fused gonads in males at IGB (gross morphology), internal melanophores in testes and renal mineralization in females at IGB (histology), as well as segmental hypoplasia, fused kidneys, dilated testicular tubules, dividing gonadocytes in testes, and one or more immature ovaries in females at WLI (histology). For several of these, the difference between two treatment means was larger than that between any one treatment and the negative control, and that appeared to be the major contributing factor to the finding of an overall difference. For others, significance levels for individual differences between treatment and control were in the range of $0.05<p<0.10$ and thus were not declared significant. Gonadal hypertrophy based upon gross morphology, and segmental aplasia based on histological findings, were found to be statistically significant overall at both labs as well. Observations of gonadal hypertrophy showed a decreasing pattern. For segmental aplasia at IGB, while no specific treatment group was shown to be significantly greater than the control group, the two highest treatment groups, 25 and 100 µg/mL, showed evidence of a significant overall trend.

Other responses which showed a significant one-sided, increasing trend were renal mineralization in males, and decreased ovarian pigmentation and decreased ovarian cavity size in females. All were observed in the IGB animals and were significant down to 1 µg/L (*i.e.* three

out of five atrazine treatment concentrations). Neither decreased ovarian pigmentation nor decreased ovarian cavity sizes were detected in the overall test for differences, however.

The data presented and summarized above were extracted from various summary and frequency tables and programs submitted in the report, checked by the Agency, and compiled for overall presentation in this paper. For the most part, statistical analyses produced consistent results between the registrant's report and EPA's evaluation, although the compilation and presentation of results may differ.

The study reports no statistical significance at either lab for gonadal deformity, gonadal segmental aplasia, gonadal translucence, decreased melanophores in ovaries, decreased germ cells in gonads, "other" renal diagnoses, increased ovarian cavity size, ovarian interstitial fibrosis, mononuclear cell infiltrates in ovary, or one or more "unable to stage ovaries." Across both labs and all atrazine treatment concentrations, the authors maintain that atrazine exposure shows no effect on growth, development, or sexual differentiation.

The authors further conclude that there are no effects of atrazine in either study on the primary study endpoints of sex ratio (frequency of males), frequency of mixed sex, frequency of intersex, or frequency of testicular oocytes. Comparisons of secondary endpoints demonstrate inconsistent and/or contradictory differences that the authors suggest are attributable to biological variability.

Analysis

In general, the studies conducted at IGB and WLI adhered, with only minor deviations, to the protocols that were submitted for review. One noted deviation was reporting atrazine concentrations only and not reporting the concentration of atrazine's major degradates, *i.e.*, diaminochloroatrazine (DACT), desethylated atrazine (DEA), and desisopropyl atrazine (DIA). Additionally, specific cell types were not enumerated during histology. Knowledge of atrazine degradate concentrations in the exposure tanks would have provided an indication of whether total measured atrazine residues (parent plus degradates) were close to nominal concentrations. Enumeration of specific cell types may have provided information on whether observations like hypoplasia were a result of a change in the actual number of cells or the volume of cells.

Although the critical window for feminization is reported by the study authors as NF Stages 42 – 54, exposures during both laboratory studies were initiated at NF Stage 48. Since the positive controls were responsive to estradiol treatment, the early development stages still appeared to be sensitive to developmental effects even though exposures did not extend through the entire window of vulnerability.

During the conduct of the study, the Agency conducted inspections of each of the study laboratories. While minor discrepancies were identified between the study protocol and the actual conduct of the studies, none of the deviations were considered critical and/or detrimental to the study. At WLI, atrazine was detected at the level of quantification (0.01 µg/L) in one cluster of the negative control tanks. The contamination was verified through LC/MS/MS, and it remained at this level in these clusters of tanks for several weeks. Atrazine was below the level of detection in the remaining control tanks. Additionally, while the protocol stated that atrazine concentrations would be measured at each test facility by ELISA and later verified by LC/MS/MS, the ELISA was abandoned prior to the initiaiton of the study due to problems with the assay, and atrazine concentrations were quantified exclusively through LC/MS/MS.

At IGB, atrazine was identified in all eight estradiol control tanks on Study Day 41. According to the report, atrazine rather than estradiol had inadvertently been added to the estradiol positive control stock solution at a concentration of 0.1 µg/L. The authors dismissed the potential for any effects due to the absence of estradiol since larvae had transitioned beyond the critical window (NF Stage 52 – 55) of vulnerability for sexual differentiation. Although the IGB report states that estradiol was not detected in any of the atrazine treatment tank solutions, no data were provided to substantiate whether atrazine treatment tank solutions were analyzed for estradiol. However, stock solutions were analyzed for estradiol and did not contain the compound. Additionally, the apical effects seen in the atrazine treatments were not consistent with those observed in the estradiol positive controls; these data suggest that estradiol contamination of atrazine treatment solutions was not an issue.

Inspections of both laboratories during the in-life portions of the study involved extensive review of raw data collection sheets and data summary tables by OECA and OPP personnel. In all cases, the data were readily traced from their point of collection to analysis. Similar to the inspections of WLI and IGB labs, EPA inspected the EPL laboratory responsible for processing tissues and histological analyses. Once again data collection sheets and data tracking efforts were

reviewed, and data were readily traced. Additionally, inspection of EPL involved the pathologist re-reading a subsample of slides to determine how readily the initial histology report could be duplicated. In all cases, the pathologist at EPL was able to replicate the initial reading; however, the severity index assigned to some of the measurement endpoints differed from the initial value, in some cases, suggesting that there is some subjectivity to the analysis. This type of subjectivity is not unique to this study and is commonly encountered in histological analyses when grades are assigned to responses.

Inspection of EPL also revealed that histology was not conducted in a completely random fashion. Due to the large number of sections requiring processing, EPL was encouraged to initiate histology prior to receiving all of the samples. Samples were received in blocks and specimens (all the sections from a single frog within the block) were randomized at EPL prior to reading It is possible that many of the specimens from WLI were reviewed by the pathologist prior to those from IGB. Additionally, it is possible that animals which metamorphosed early were reviewed prior to those animals which metamorphosed later. The extent to which this bias occurred is uncertain, and it is also uncertain whether it introduced "drift" in the pathologist's review. It is noteworthy, though, that the inspection also indicated that the pathologist was able to reproduce pathology reviews when requested to re-evaluate the sections from selected frogs.

Exposure tank environmental conditions in the two laboratories did not deviate significantly across treatments. (Mean values are presented in **Appendix 2**.) Ammonia levels in both laboratories showed similar trends over time where concentrations increased gradually to a maximum between study days 30 – 40 and then declined rapidly afterward. The increase in ammonia levels corresponded to increases in larval growth (loading rate), and the decline in ammonia coincided with decreases in loading rates as animals metamorphosed and were removed from the exposure aquaria. The maximum measured ammonia concentration was 1.4 mg/L, which is well below the concentrations of concern discussed in the original White Paper (USEPA 2003).

The most notable difference between environmental conditions in the two laboratories involved pH values; pH in aquaria at IGB was significantly ($p<0.001$) correlated with the sampling date. At IGB, pH steadily increased (pH range 7.5 – 8.5) over the entire sampling period; whereas at WLI, pH fluctuated but there was no apparent trend across the study period. The pH range, though, was within acceptable levels. Growth and development reported in this

study were consistent with normal development values reported for *X. laevis* (Nieuwkoop and Faber 1994) and suggest that environmental conditions had been reasonably optimized to promote the development and growth of the study animals.

Dissolved oxygen (DO) concentrations over the study period showed a similar pattern in both laboratories. Mean daily DO concentrations began to decline from the start of the WLI study and continued to decline until Day 27. Supplemental oxygen was initiated on study Day 28 after which time DO began to increase over the following study days to around 8.5 mg/L. The lowest mean daily DO measured at WLI was 6.0 mg/L (approximately 68% saturation at 22^{o}C). At IGB, DO concentrations began to decline around Day 10 of the study and reached a low of 6.5 mg/L (approximately 75% saturation) at around Day 20 of the study. Similar to what was observed at WLI, DO concentrations at IGB began to increase in exposure tanks once supplemental aeration was provided.

Measured concentrations of atrazine and of estradiol deviated from nominal concentrations. **Table 18** provides the range of measured concentrations along with the percentage of nominal measured concentrations represented. Although measured concentrations deviated from nominal, the mean measured atrazine concentrations and the average percent of nominal across each of the treatment groups were consistent with nominal concentrations for WLI with percent of nominal ranging between 87 – 112% (**Table 18**). Mean measured concentrations of atrazine at IGB showed greater variability, with the percentage of nominal ranging between 55 – 88% (**Table 18**). The frequency of measurements, though, allows actual exposure concentrations to be tracked throughout the course of the study. Although measured concentrations were not equivalent to nominal, the exposure concentrations did span several orders of magnitude and bracketed concentrations where effects on amphibian gonadal development have been reported in previous studies (Hayes *et al*. 2002; Hayes *et al*. 2003; and Carr *et al.* 2003). Since measured concentrations in stock solutions were consistent with nominal, and flow metering/delivery devices were carefully monitored throughout the study, the loss of both atrazine and estradiol in the treatment tanks likely is attributed to uptake by the test animals. This is consistent with the observations that as animals were removed from the tank following completion of metamorphosis, measured concentrations better approximated nominal concentrations.

Table 18. Summary of mean-measured concentrations (µg/L) of atrazine across treatment groups and study laboratories.

Nominal Concentration	Wildlife International		Leibniz-Institute of Freshwater Ecology and Inland Fisheries	
	Mean ± Std Error (Range) µg/L	Average Percent of Nominal (Range)	Mean (Range) µg/L	Average Percent of Nominal (Range)
0.0	<0.01	--	<0.01	--
0.01	0.011 ± 0.001 (0.006 – 0.018)	112% 60 – 180%	0.0069 ± 0.0008 (0.004 – 0.016)	68.6% 40 – 160%
0.1	0.092 ± 0.003 (0.062 – 0.110)	92.4% 62 – 110%	0.055 ± 0.005 (0.022 – 0.110)	55.4 22 – 110%
1.0	0.957 ± 0.019 (0.680 – 1.10)	95.7% 68 – 110%	0.716 ± 0.035 (0.430 – 1.00)	71.6 43 – 100%
25	23.4 ± 0.75 (18 – 29)	93.6% 72 – 116%	22.0 ± 0.73 (16 – 28)	88.2% 64 – 112%
100	86.7 ± 2.91 (67 – 109)	86.7 67 – 109%	87.9 ± 3.86 (61 – 120)	87.9% 61 – 120%

In general, estradiol appeared to serve as a reasonable positive control in the study. Study animals were responsive to the chemical, and the concentration of estradiol tested (0.2 µg/L) resulted in a conversion of roughly 50% of the males to females (sex ratio of male to female of 25:75). Effects observed in the estradiol control animals were consistent with what have been reported in previous studies in terms of feminization of the gonads. There were, however, effects observed in atrazine-treated animals (decreased ovarian cavity size and decreased ovarian pigmentation) along with borderline effects for fused gonads, renal mineralization, and fused kidneys that were either not observed or were at 'borderline' significance (*i.e.*, decreased ovarian cavity size) in estradiol-treated animals. Similarly, there were numerous effects observed in estradiol-treated animals that were not observed in atrazine-treated animals. It is important to note that estradiol was used as a putative positive control since it is known to affect gonadal development of amphibians and is not intended to represent a common mechanism of action to atrazine.

The study authors claim that across both labs and all treatment concentrations, atrazine exposure showed no effect on the apical study endpoints of time to and size at metamorphosis (growth), development (presence of mixed and/or intersex animals), or sex ratio (sexual differentiation) (Hosmer *et al.* 2007). Although there were no effects of atrazine shown in either study on the primary study endpoints of concern, *i.e.*, sex ratio (frequency of males), frequency of mixed sex, frequency of intersex, or frequency of testicular oocytes, significant differences for a number of secondary gonadal effects were demonstrated in both labs. While their relation to

primary apical endpoints is not known, the possibility that some of these secondary measurement endpoints may serve as precursors to primary effects or point to other endpoints is uncertain.

One of the histological measurement endpoints, *i.e*., decreased germ cells, was reported as decreased in both control and atrazine-treated animals. It is unclear how both control and treated animals can exhibit a decrease since presumably the treated animals are being compared to the controls. This suggests that for this measurement endpoint, comparisons were not made against the study controls but rather some other reference group. In follow-up discussions with the registrant, for characterizing the relative abundance of germ cells, the pathologist relied on his understanding of what is typical for fish gonads (personal communication: Alan Hosmer, Study Director, Syngenta 2007). Since *X. laevis* gonad maturation is not complete at metamorphosis and is likely dependent on the chronological age of the animal rather than on the animal's external developmental NF stage, the relative abundance of germ cells may have little practical use particularly when the observation is made relative to fish as opposed to control animals.

The authors also mention comparisons of secondary effects that demonstrated inconsistent and/or contradictory differences between laboratories and suggest they are attributable to biological variability (Hosmer *et al.* 2007). In the current study, each animal represented a proportion (and increase or decrease) of roughly 8%, on average, and higher when fewer animals were present per tank. Because of this, the data demonstrated considerable variability from tank to tank, with differences being caused by a relatively small number of animals. For gross morphological endpoints, in particular, the Agency acknowledges that the less than definitive nature of gross inspection compared with histological diagnosis may have combined with biological variability to produce apparent inconsistencies in the findings between laboratories on some endpoints. Given this variability in the data, however, along with the small numbers of animals and rates of responses involved for certain endpoints that were analyzed by sex, combined with the loss of controls at the WLI lab, it is not surprising that low incidence findings in one lab are not always demonstrated in the other. Despite these considerations, however, as noted above, certain effects were demonstrated in both positive control and atrazine-treated groups, and the results of the study should be interpreted in light of this.

Appendix 2 contains tables of summary statistics and analysis results conducted by the Agency on the registrant's data. The results of the Agency's analysis were consistent with those found in the registrant's report.

With respect to the evaluation criteria, each of the DCI studies contained deviations from the study protocol in terms of contamination of controls and measured versus nominal exposure concentrations. Additionally, there are uncertainties regarding the relevancy of some of the measurement endpoints and the process used for evaluating these endpoints. However, in general the studies addressed the design elements identied in the Agency's 2003 White Paper and in doing so accounted for potential sources of variability that limited previous studies. The high degree of quality control associated with these studies provided a means to surmount the major protocol deviations and quantitatively analyze the study results. Based on the apical endpoints measured in these studies, atrazine exposure of *X. laevis* from NF Stage 46 through NF stage 66 does not affect gonadal development or sex ratio. Additionally, there was no consistent dose-respone of atrazine on time to metamorphosis or size at metamorphosis.

CHAPTER 3

OPEN LITERATURE STUDY REVIEWS

Subsequent to the 2003 SAP, a number of laboratory and field studies have been published in open literature on the effects of atrazine on amphibian gonadal development. Many of these studies represent the final published versions of interim reports initially reviewed by the Agency and summarized in its 2003 White Paper.

In this chapter, summary reviews, grouped into laboratory and field studies, are presented for each individual study. Each study was individually evaluated with regard to experimental design, protocols and data quality assurance, strength of cause-effect and/or dose-response relationships, mechanistic plausibility, and ecological relevancy of measured endpoints. Throughout the reviews, the terms intersex, hermaphroditism, and ovotestes were used interchangeably and refer to situations where ovarian and testicular tissue are observed in the same animal/gonad.

Consistent with EPA's process for evaluating scientific studies, the Agency completed data evaluation records (DERs) for those studies where raw data were available and conducted a critical review of the pertinent studies reported in the open literature. It is important to note that the review of registrant-sponsored studies is typically more detailed than for studies obtained from open literature. For these latter studies, the reviews are generally less detailed because EPA does not have access through the study authors to the full range of raw data and quality control information required for registrant-submitted GLP studies.

Laboratory Studies

As noted in the 2003 White Paper (USEPA 2003), the major advantage in conducting laboratory studies is that they allow researchers to control a range of variables, which could potentially impact the outcome of a study. Environmental factors, water quality, loading rate, chemical exposure, study animals, animal husbandry and health can all be manipulated more easily to identify actual treatment effects in laboratory studies. Laboratory studies also allow greater flexibility in study design to account for known sources of variability. For example, sample size and replication can be manipulated to reduce confidence intervals around treatment means. Another advantage of laboratory studies is that researchers can use positive controls to gauge the responsiveness of the test organisms to treatment effects. Finally, laboratory studies facilitate sample processing since analyses can be conducted in close proximity to where samples are collected.

Since the 2003 SAP was held nine laboratory studies (identified in Table 1) were published in the open literature that examine the effects of atrazine on amphibian gonadal development. Of the nine laboratory studies published in the open literature, two (*i.e.*, Coady *et al.* 2004 and Coady *et al*. 2005) represent the final published versions of studies that were reviewed as interim reports in the 2003 White Paper. For those studies that were previously reviewed, only a brief synopsis of the final paper is provided below. A more thorough description of the study, its results, and the reviewers' analyses can be found in **Appendix 1**.

Freeman, J. L. and A. L. Rayburn. 2005. Developmental Impact of Atrazine on Metamorphing *Xenopus laevis* as Revealed by Nuclear Analysis and Morphology. *Environmental Toxicology and Chemistry* 24(7): 1648 – 1653.

Description and Results of Study

The purpose of this study was to determine if exposure of developing African clawed frogs (*Xenopus laevis*) to environmentally relevant concentrations of atrazine alters metamorphosis. *Xenopus* tadpoles were obtained from commercial suppliers (NASCO, Fort Atkinson, WI, and Xenopus Express (Plant City, FL). Stock solutions were prepared using technical grade atrazine (98% active ingredient) in water and atrazine concentrations in stock solutions, and exposure tanks were verified using ELISA (Abraxis, Ivyland, PA). Tanks were maintained at 22^{o}C, and water quality (pH, nitrites, ammonia, and dissolved oxygen) was monitored "routinely".

In the first study, Nieuwkoop Faber Stage 47 larvae were individually exposed to 0, 200, 400, 600, or 800 ppb atrazine in 3-L tanks; each treatment group was replicated 3 times. Each treatment consisted of a single tank, and water was changed once per week. After one, two, and three weeks of exposure, larvae were prepared for nuclear DNA analysis using an EPICS XI flow cytometer (Coulter Electronics, Hialeah, FL) and a protocol by Gold *et al*. (1991). In a second study, NF Stage 47 larvae were individually exposed to either 0 or 800 ppb atrazine per 3-L tank for 3 weeks; each treatment was replicated 6 times. In a third study, NF Stage 54 larvae were exposed to either 0 or 800 ppb atrazine, and the study was replicated 7 times. In a fourth study, no exposure to atrazine occurred; rather, tadpoles were chosen at random from 38-L tanks, staged and weighed, and analyzed for the number of nuclei. In a fifth study, two NF Stage 40 – 42 larvae were exposed together to either 0 or 800 ppb atrazine in 3-L tanks; tadpole development was quantified by staging, weighing, and nuclei analysis after one, two, three, four, and five week exposures; this study was replicated 5 times. In a sixth study, five NF Stage 52 – 53 were placed in 8-L tanks and exposed to 0, 100, 450, or 800 ppb atrazine for 4 weeks until metamorphosis (NF Stage 66) at which point the tadpoles were staged, weighed, and prepared for nuclei counting; the study was replicated 2 times. Statistical analyses involved comparing coefficients of variation

According to the authors, pH ranged between 7.4 to 7.8, nitrite and ammonia levels were around 0 mg/L, and dissolved oxygen ranged between 8 to 10 mg/L throughout all of the studies.

In the first study, mortality was 9.3% and occurred randomly among treatments. After three weeks of the study, there was no statistical difference for cytometric flow analyses between the treatments. In the second and third studies where NF Stage 47 and NF Stage 54 larvae were exposed to either 0 or 800 ppb atrazine for 3 weeks, there was a significant difference between cytometric flow CVs for control and atrazine-treated larvae with p=0.0181 and p= 0.0471, respectively for each of the two studies.

In the fourth study, nuclei counts and body weights were "calibrated" with the developmental stage (**Table 19**). Larvae increased in weight and nuclei count until reaching NF Stage 57 and 58. At Stage 59 when tadpoles initiated metamorphosis, body weight and number of nuclei decreased until metamorphosis was complete at NF Stage 66.

Table 19. Developmental stages and associated mean number of nuclei and body weights.

Developmental Cluster Group	NF Stage	Total Number of Tadpoles	Mean Total Number of Nuclei	Mean Weight (g)
No legs	47	8	2.0×10^6	0.31
Early 2 legs	48 – 51	34	4.0×10^6	0.58
2 legs	52 – 55	13	5.3×10^6	0.74
Late 2 legs	56 – 57	20	6.1×10^6	0.89
4 legs	58 – 62	9	3.1×10^6	0.88
4 leg metamorph with tail	63 - 64	6	1.9×10^6	0.58
4 leg metamorph no tail	65 - 66	4	2.6×10^6	0.65

In the fifth study where NF Stage 40 – 42 larvae were exposed to either 0 or 800 ppb atrazine and analyzed after 1, 2, 3, 4, and 5 weeks, there was no significant difference for nuclei counts between control and 800 ppb after 1, 2, 3, or 4 weeks of exposure. Stages were also not significantly different after the first 4 weeks of exposure. However, after 5 weeks of exposure, the nuclei counts and developmental stage were both significantly different (p=0.0066; n=6) from the controls; tadpoles in the 800 ppb treatment were at a younger stage of development (NF Stage 55) compared to controls (NF Stage 65). Weights were not significantly different at any week. There was no difference in mortality at approximately 5.6%

In the sixth study where NF Stage 52 – 53 were exposed to a range of atrazine concentrations, there was no significant difference for nuclei counts (p=0.2964) or weights (p=0.2107) for atrazine treated versus controls. Stages were found to be significantly different (p=0.0297); all three atrazine exposures were different and at earlier stages compared to controls (**Table 20**). Tadpoles at the youngest stage of development were treated with the highest

concentration of atrazine, *i.e.*, 800 ppb. A single tadpole died during this study in the 100 ppb atrazine treatment.

Table 20. Mean total number of nuclei and associated developmental stage of larvae exposed to increasing concentrations of atrazine.

Atrazine Conc (ppb)	N	Total Number of Animals	Mean Total Number of Nuclei	Mean NF Stage	Mean Weight (g)
0	2	10	3.6×10^6	63	0.63
100	2	9	5.3×10^6	56*	0.71
450	2	10	6.2×10^6	57*	0.82
800	2	10	4.6×10^6	52*	0.66

*statistically different at $p<0.05$

The study authors concluded that 100 ppb is the lowest concentration of atrazine at which a significant effect on development was seen. According to the authors, atrazine increased time to metamorphosis when exposure was initiated pre-metamorphosis, and they hypothesized that atrazine disrupts normal thyroid function which controls the rate of metamorphosis.

Analysis

As background, according to Nieuwkoop and Faber (1994), NF Stage 40 – 42 larvae are 2 – 3 day post-hatch. NF Stage 47 corresponds to roughly 5-days post-hatch.

Although the study reports that larvae were exposed individually in 3-L containers, it does not state the fill volume or the composition of the containers. No information is provided on whether the tadpoles were fed during the exposure period and if so, what the food consisted of. No information is provided on whether the exposure tanks were aerated. By the 6th study, up to 5 NF stage 53 larvae were placed in 8-L containers and maintained for 4 weeks until metamorphosis (NF Stage 66) which, according to Nieuwkoop and Faber (1994), typically occurs around 58 days post-hatch.

Exposure concentrations of atrazine (100 – 800 ppb) are relatively high compared to what has typically been evaluated in preceding studies.

In the third study, the authors describe the NF Stage 54 tadpoles as having hind limbs present but no foot; however, the authors then proceed to describe the appearance of the foot and its digits. According to Nieuwkoop and Faber (1994), at Stage 54 (26-days post-hatch), a foot is present and all four digits can be distinguished.

In the 6th study, NF Stage 53 (24 days post-hatch) larvae were exposed to atrazine until NF-stage 66 (metamorphosis) (58 days post-hatch). According to the study, it took 4 weeks (28 days) for the larvae to complete metamorphosis. The total time (24+28=52 days) is relatively consistent with the average time (58 days) reported in Nieuwkoop and Faber (1994).

It is unclear why statistical comparisons of coefficients of variation (CV) were run rather than simply testing actual means.

Although the study authors hypothesize that the increased time to metamorphosis associated with atrazine exposure is indicative of the chemical disrupting normal thyroid function, the authors did not measure thyroid activity in their study and as such, their hypothesis is untested.

Animal husbandry is not well explained in the study nor is it explained how eggs from two different sources were distributed in the study. Since feeding is not discussed, it is unclear whether food was a limiting factor in the study. However, mortality was low in each of the different experiments, and development rates among controls were consistent with those estimated by Nieuwkoop and Faber (1994). Although delayed development was observed in the studies, there was no difference in the size of the animals.

The analysis following exposure to a range of atrazine concentrations (100 – 800 ppb) revealed larvae at significantly earlier development stages in atrazine-treated groups compared to controls; the implication is that atrazine-treated animals could take longer to complete metamorphosis. However, this hypothesis is untested since the study did not measure time to complete metamorphosis. Additionally, the lower developmental stage of atrazine-treated larvae did not appear to be a monotonic dose response.

The study does not provide much detail on the culture conditions for each of the studies; however, loading rates and water quality appeared to be sufficient to promote reasonable growth in controls and minimize mortality. No data are presented to determine whether controls were monitored for atrazine or whether the feed used in the study was monitored for atrazine or phytoestrogens. There did not appear to be a consistent dose-response; however, this study indicates that at the lowest concentration tested, atrazine exposure delayed the development of *X.*

laevis. The study does not provide any information of the potential effects of atrazine on amphibian gonadal development. Although the study indicates that larvae exposed to different concentrations of atrazine were at statistically significant earlier stages of development, the body weights of these animals were not statistically different. While atrazine-exposed animals were at earlier stages of development than controls, it could be presumed that atrazine-exposed animals require longer to complete metamorphosis; however, the study did not measure time to complete metamorphosis. Since time and size at metamorphosis were not measured in this study, the study has limited value in determining if atrazine affects metamorphosis of African clawed frogs.

Hayes, T. B., A. A. Stuart, M. Mendoza, A. Collins, N. Noriega, A. Vonk, G. Johnston, R. Liu, and D. Kpodzo. 2006a. Characterization of Atrazine-induced Gonadal Malformations in African Clawed Frogs (*Xenopus laevis*) and Comparisons with Effects of an Androgen Antagonist (Cyproterone Acetate) and Exogenous Estrogen (17β- Estradiol): Support for the Demasculinization/Feminization Hypothesis. Environmental Health Perspectives 114 (Supplement 1): 134 – 141.

Description and Results of Study

This study is intended to address the hypothesis that gonadal malformations induced by atrazine represent "demasculinization" or loss of androgens, and/or one or more of the types of gonadal malformations represent feminization as a result of "inappropriate" estrogen production in African clawed frogs (*Xenopus laevis*). Adult *X. laevis* (3 males and 3 females) were obtained from a captive colony maintained at the University of California, Berkeley (CA). Study units consisted of covered plastic mouse boxes containing 4 L of 10% Holtfreter's solution and 30 larvae (4-days post-hatch). All tanks were aerated and were maintained at 22$\pm$1^{o}C with a 12:12 hr light:dark cycle.

Dilution water was analyzed for a variety of pesticides, including atrazine and N-triazine degradates and determined not to have detectable residues. Animals were fed a solution of homogenized Purina® rabbit chow *ad libitum*. All treatments were replicated 3 times and were color coded (blinded). Tanks were cleaned, and the exposure solutions completely renewed every three days except in the estradiol study (Experiment 2) where exposure solutions were renewed every day. At completion of metamorphosis (Nieuwkoop Faber Stage [NF] 66), animals were removed from tanks and euthanized with 0.2% benzocaine, weighed, measured and then fixed in Bouin's solution for histological analysis. In the first of two experiments, *Xenopus* larvae were treated with cyproterone acetate (CPA) (5 mg/L dissolved in methanol) throughout development (NF Stage 50 – 66). Methanol concentrations in each of the treatments were 0.004%; additionally, solvent controls and negative controls were run.

In a second experiment, *Xenopus* larvae (NF Stage 50) were treated with 17β-estradiol (100 µg /L dissolved in ethanol) for 7 days (NF stages 50 – 53), 14 days (NF stages 50 – 55), and 49 days (NF stages 50 – 66). Exposure solutions were changed daily; each exposure solution contained ethanol at 0.004%; solvent controls and negative controls were also run. Animals sacrificed at NF Stage 66 were euthanized and then fixed in Bouin's solution.

The paper states that a subset of animals (n=150) was prepared for histological analysis. Anomalies were defined as gonadal malformations that were not observed in controls and the terminology was based on anomalies observed in 30 of the animals treated with 25 ppb atrazine.

Oocytes were not observed in the ovaries of any of the female animals, including NF Stage 66 females, under the study conditions tested. None of the solvent control and negative control animal sex ratios deviated from the expected 50:50:0 (male:female:malformed gonads) ($p>0.50$). According to the authors, there were no tank effects among controls for sex ratio; control groups from across the three experiments were homogeneous.

Gonadal anomalies observed in atrazine-treated animals included "single-sex polygonadalism" (SSP), consisting of broken, lobed, or discontinuous testes and hermaphroditism where gonads consisted of multiple combinations of testes and ovaries. Nonpigmented ovaries were the most common abnormality among atrazine-exposed animals, and SSP was the second most common malformation. The frequency of malformations varied between concentration, and the highest number of total malformations occurred in the 1 ppb atrazine treatment. The highest frequency of unpigmented ovaries occurred in the 0.4 ppb treatment, whereas SSP and hermaphroditism occurred in the highest frequency in the 1 ppb atrazine treatment. According to the authors, atrazine caused a significant ($p<0.001$) increase in the number of malformations in all cases. Although malformations were originally defined by their absence in control animals, a reanalysis indicated nonpigmented ovaries in 3 of the methanol and 1 of the ethanol solvent controls.

Exposure to CPA resulted in a 36% incidence of nonpigmented ovaries that were, according to the authors, similar to the morphology "produced" by atrazine.

Exposure to estradiol for the entire larval period resulted in 100% females based on gross morphology and confirmed by histology; no SSP or hermaphroditism was reported in the NF Stage 66 animals that had been exposed to estradiol for 49 days. Larvae treated for 7 days or 14 days showed SSP and hermaphroditism, which the authors characterized as incomplete sex reversal. The highest frequency of malformations occurred in the 7-day treatment. Nonpigmented ovaries were not observed among animals treated with estradiol.

According to the authors, the [gonadal] malformations induced by atrazine in previous studies (Hayes *et al.* 2002 and Carr *et al*. 2003) were reproduced in the present study by exposure to CPA (unpigmented ovaries) or exposure to estradiol (hermaphroditism and SSP), and supports the hypothesis that atrazine-exposed larvae "suffer from a combination of androgen reduction and increased estrogen production" potentially as a result of induction of aromatase.

Testicular oocytes were not observed in any of the treated or control animals. The authors note that testicular oocytes reported in Jooste *et al.* (2005) have only previously been reported following exposure of larvae to "near-lethal" feminizing temperatures (10^{o}C) during critical stages of gonadal development.

Analysis

The study design contains a number of deficiencies. Purities of the atrazine, cyproterone acetate, and 17β-estradiol are not stated. Because the studies were conducted in plastic rodent containers rather than in glass or Teflon-lined containers, it is uncertain whether plasticizers, *e.g*., phthalates, may have leached from the plastic into the exposure solutions. The loading rate (30 larvae/4 L) reported in the study exceeds the ASTM recommended rate of 1 larvae/L, and pesticide or phytoestrogens analyses are not reported for the Purina® rabbit chow.

One of the largest uncertainties is whether larvae were exposed to atrazine concentrations of 0.1, 0.4, 0.8, 1.0, and 25 ppb as part of the current study, or whether the exposure took place during a previous study, and histology samples from the previous studies were compared against current treatments with cyproterone and estradiol. Since only two experiments are identified in the study, it suggests that the atrazine study took place earlier and did not rely on the same three breeding pairs of adult *Xenopus* used as a source of larvae in this study.

The paper states that a subset of animals (n=150) was prepared for histological analysis; however, no mention is made of how the subset was determined. The authors state that initially, gonadal malformations were defined as any gonadal anomaly not observed in controls. Later, they state that the frequency of malformations was determined in controls and in animals treated with a range of atrazine concentrations.

The paper does not mention whether exposure concentrations were verified through analytical measurements. Additionally, the paper does not describe how sex ratios were determined. Specifically, the report does not indicate whether the sex of all animals was determined through gross morphology and/or through histology since only a subsample of animals were used for histology.

It is also unclear from the information presented on sex ratio whether animals identified with "malformed gonads" represent those animals where gender could not be distinguished and are either mixed sex or simply unknown.

The study authors cite a study by Carr *et al* (2003) which describes similar malformations at similar frequencies induced by similar atrazine concentrations in *X. laevis*. The Agency reviewed the work by Carr *et al*. (2003) (USEPA 2003) and found that it had significant problems associated with animal husbandry that seriously limited the utility of the study in establishing a causal relationship between atrazine exposure and gonadal developmental effects. Additionally, the authors cite the work by Tavera-Mendoza *et al*. (2002a and 2002b) as being supportive of their current findings. Again, the Agency has reviewed these studies and determined that they also have limited utility to refute or confirm whether atrazine exposure can affect amphibian gonadal development (USEPA 2003).

The authors report the absence of testicular oocytes in their study; however, the relevancy of this information to testing their hypothesis is unclear since they report single sex polygonadalism. The study authors cite Jooste *et al.* (2005), a study in which as many as 57% of atrazine-treated and control males had testicular oocytes. However, as discussed later in this White Paper, the Agency has reviewed the mesocosm study by Jooste *et al.* (2005) and has determined that the study has limited utility in estimating the potential effects of atrazine on amphibian gonadal development.

The authors indicate that single sex polygonadalism was only observed in males. This fact along with other lines of evidence are considered by the authors to be indicative that only males are affected and as such, hermaphrodites represent demasculinized/feminized males as opposed to masculinized/defeminized females. Based on the figure depicting the frequencies of males, females, and metamorphs with gonadal malformations (see top of **Figure 2** representing Figure 9 B of the monograph), the percentage of females across all atrazine treatments and the

control do not appear to deviate substantially from 50% except in the 0.4 ppb atrazine treatment group where the number of females is reduced. Additionally, the percentages of gonadal anomalies among atrazine-exposed NF-stage 66 animals does not appear to be related to dose for the anomalies taken together or individually. It is unclear from the paper whether the nonpigmented ovaries, accounted for in Figure 9B of the monograph (**Figure 2** below), are intact ovaries of presumably female animals or whether they are intended to represent nonpigmented ovarian-like tissue in indistinguishable gender animals. According to the paper, though, they appear to be "ovaries."

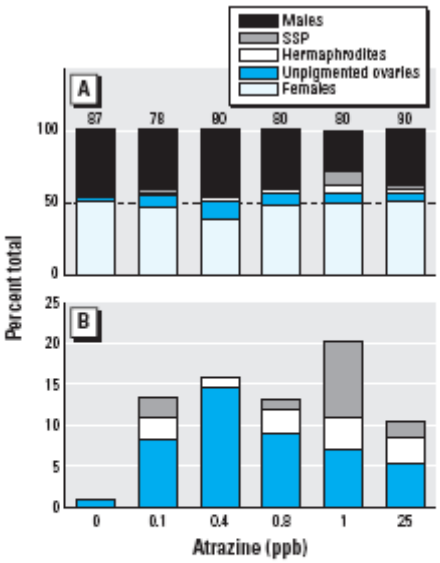

Figure 9. (*A*) Frequency of males, females, and specimens with gonadal malformations in controls and atrazine-treated animals (0.1–25 ppb). Numbers above bars are samples sizes and represent the number surviving to metamorphosis (of 90). Dashed line indicates 50%. (*B*) Frequency of gonadal malformations only. *X*-axis is categorical.

Figure 2. Figure 9 from Hayes *et al.* (2006a).

The study contains findings that are difficult to interpret from the information presented in the paper. There does not appear to be a consistent dose-response relationship between atrazine concentrations and the incidence of gonadal malformations. It is not clear whether animals with gonadal malformations such as nonpigmented ovaries remained unclassified in terms of gender in calculating sex ratios. The relevancy of nonpigmented ovaries is also unclear since histology indicated that the gonadal tissue was clearly ovarian. According to the figure depicting results from the CPA exposure (**Figure 3** depicting Figure 11 of the Hayes *et al.*(2006) monograph), roughly 10% of the animals are reported as females, roughly 55% are reported as

nonpigmented ovaries, and the remainder is reported as male. If the ovaries can be distinguished this way, it is unclear why the sex ratio isn't simply reported as approximately 65:35 (female:male).

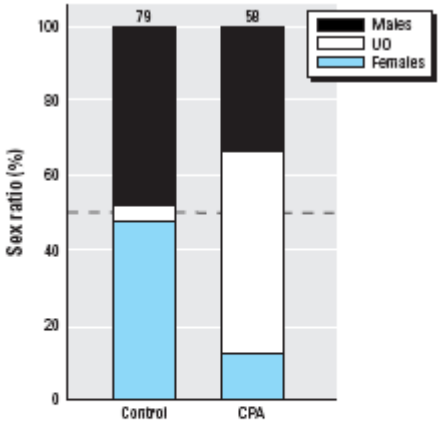

Figure 11. Frequency of males, females, and animals with nonpigmented ovaries in animals exposed to CPA compared with solvent (methanol)-treated controls. Numbers above bars are samples sizes and represent the number surviving to metamorphosis (of 90). Dashed line indicates 50%.

Figure 3. Figure 11 from Hayes *et al.* (2006a).

In the figure depicting the sex ratios of larvae exposed to estradiol for differing periods of time, the control animals appear to have a sex ratio of roughly 35:65 (female:male) (**Figure 4** depicting Figure 13 of the Hayes *et al.*(2006) monograph). It is unclear why the sex ratio of the controls does not better approximate 50:50. It is also unclear whether the controls depicted in **Figure 4** represent those exposed 7 days (NF stages 50 – 53), 14 days (NF stages 50 – 55), or 49 days (NF stages 50 – 66) since the animals are at different developmental stages.

As with many studies, the authors have attempted to define terms for describing various histological effects that have been observed over a range of study conditions. It is unclear whether the authors' terminology and/or exposure conditions are comparable to those used in other studies (Carr *et al.* 2003; Hecker *et al.* 2003; Gross *et al*. 2003; Smith *et al*. 2003; Du Preez *et al*. 2003). It also appears that the current authors have not replicated their own previous work reported in Hayes *et al*. (2002) and Hayes *et al*. (2003).

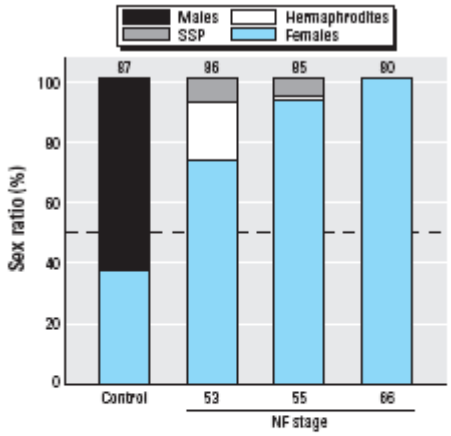

Figure 13. Frequency of males, females, animals with SSP, and hermaphrodites in animals treated for 7, 14, or 49 days with 100 µg/L E_2. *X*-axis is categorical. Control bar shows the control sex ratio. NF stages below the bars indicate the stage at which E_2 exposure was terminated. All E_2 exposures began at NF stage 50 (Figure 2). Numbers above bars are samples sizes and represent the number surviving to metamorphosis (of 90). Dashed line indicates 50%.

Figure 4. Figure 13 from Hayes *et al.* (2006a).

This study is consistent with the previous studies of *X. laevis* in which the authors demonstrated that atrazine exposure affects gonadal development. However, the authors continue to rely on methods (*e.g.,* plastic mouse cages as exposure units; high loading rates) that have previously been identified as sources of variability and uncertainty. One of the major problems of this study is that the methods are not reported, and it is possible that the data are from a previous study or from an unreported aspect of the current study. Additionally, it appears that atrazine and/or phytoestrogen were not measured in the feed, and water quality parameters were not reported in the study. Similar to previous studies, the measurement endpoints do not exhibit a clear dose-response relationship. Sex ratio reported for some of the control animals (35:65) appear to be skewed from the expected value of roughly 50:50. Finally, the relevancy of some of the measurement endpoints such as unpigmented ovaries is unclear and is inconsistent with the authors' observation that atrazine exposure primarily affects male gonadal development.

Hayes, T. B., P. Case, S. Chui, d. Chung, C. Haeffele, K. Haston, M. Lae, V. P. Mai, Y. Marjuoa, J. Parker and M. Tsui. 2006b. Pesticide Mixtures, Endocrine Disruption, and Amphibian Declines: Are We Underestimating the Impact: Environmental Health Perspectives 114 (Supplement 1): 40 – 50.

Description and Results of Study

The objective of this study is to examine the effects of four herbicides (atrazine, metolachlor, alachlor, and nicrosulfuron), three insecticides (cyfluthrin, cyhalothrin, and tebupirimphos) and two fungicides (metalaxyl and propioconizole) alone and in combination on size, and time to metamorphosis, gonadal differentiation, and thymus histology as a measure of immunocompetence in the Northern leopard frog (*Rana pipiens*). Atrazine, alachlor, nicrosulfuron, cyfluthrin, λ-cyhalothrin, tebupirimiphos (97% active ingredient), metalaxyl, and propioconizole were obtained from Chem Service, Inc. (Chester, PA) with a purity of >98% active ingredient. The remaining chemicals were obtained from Syngenta Crop Protection (Research Triangle Park, NC).

Experiment 1: Effects of Pesticides on Larval R. pipiens

Adult *R. pipiens* (3 males and 3 females) were obtained from Sullivan Co., Inc., (Nashville, TN) and were induced to spawn. Eggs were maintained in 10% Holtfreter's solution. Two days post-hatch, 30 free-swimming larvae (Gosner stage 21) were apportioned to each exposure chamber, consisting of "plastic mouse boxes" that contained 4 L of 10% Holtfreter's. Tanks were aerated, and larvae were fed Purina® rabbit chow; food levels were adjusted as the larvae grew.

Larvae were treated with each of the pesticides alone at 0.1 ppb, with mixtures of all the pesticides together (each at 0.1 and 10 ppb each), with atrazine plus S-metolachlor at 0.1 or 10 ppb each, or with the formulated product Bicep II (atrazine plus S-metolachlor) at a rate equivalent to 0.1 or 10 ppb of atrazine in the mixture. All pesticides were predissolved in ethanol; final concentration of ethanol was 0.0036% in each of the treatments and in the solvent control. A negative control consisted of 10% Holtfreter's solution alone. The study was replicated 3 times. Cages were cleaned, and the exposure solution was changed every 3 days.

Exposures continued from Gosner stage 21 to Gosner stage 46 (completion of metamorphosis). Samples for chemical analysis were taken just after making up the test solutions. At completion of metamorphosis, each animal was weighed and measured (snout-to-vent length; SVL); animals were euthanized in 0.2% benzocaine and then fixed in Bouin's solution. Histology sections were cut at 8 μm. In cross-sections of the thymus, the number of thymic plaques, maximum transverse cross-sectional area and cell density were determined. Animals exposed to the mixture of atrazine and S-metolachlor did not undergo histological analysis of the thymus.

Experiment 2: Effects of Pesticide Mixtures on Corticosterone Levels in Adult African Clawed Frogs (*Xenopus laevis*)

Adult male African clawed frogs were obtained from a long-term captive colony maintained at the University of California (Berkeley, CA). Animals were housed individually and acclimated to 10% Holtfreter's solution for 5 days. Afterward, 5 animals were exposed to the pesticide mixture at 0.1 ppb (each pesticide), and 5 animals were exposes to a solvent control (0.0036% ethanol). Tanks were cleaned and treatment solutions were renewed every 3 days. Animals were fed a diet of Purina® Trout Chow daily. Exposure continued for 27 days. Blood was collected by cardiac puncture between 1800 and 2000 hr, centrifuged at low speed, and the plasma stored at -80^{o}C. Corticosterone analysis was conducted by radioimmunoassay.

For exposure solutions, the limit of detection for all of the pesticide analyses in water was 0.1 ppb. Atrazine (0.19 ppb), alachlor (0.15 ppb), S-metolachlor (0.22 ppb), metalaxyl (0.16 ppb), and propioconazole (0.23 ppb) were measured in the 0.1 ppb treatments, while no pesticides were detected in the Holtfreter's control (<0.1 ppb).

In Experiment 1 according to the report, mortality ranged from 0 – 7.8% for animals exposed to a single pesticide, and there were no statistical differences in mortality across treatments ($p>0.05$), except among larvae treated with metalaxyl where mortality was 36%. One of the replicates in the metalaxyl group was dropped because 90% of the animals died after the loss of aeration. Larvae exposed to the mixture of atrazine plus S-metolachlor or Bicep II experienced <10% mortality; however, in the mixture of all the pesticides at 0.1 ppb, mortality was 34% up to Gosner stage 42 (forelimb emergence). Mortality in animals exposed to the mixture of all the pesticides at 10 ppb died during the first day of exposure.

Time to metamorphosis and SVL were not statistically different across individual pesticide treatments relative to controls except for animals treated with propioconazole. Animals treated with propioconazole took significantly ($p<0.0001$) longer to initiate metamorphosis (forelimb emergence at Gosner stage 42) and complete metamorphosis (tail resorption at Gosner stage 46).

According to the paper, animals exposed to pesticide mixtures at 0.1 ppb had significantly longer larval periods. Initiation of metamorphosis ($p<0.005$) and completion of metamorphosis ($p<0.0001$) were significantly delayed. Larvae exposed to the nine-compound mixture were similarly delayed ($p<0.0001$).

Size at metamorphosis was significantly ($p<0.05$) lower than controls for animals treated with atrazine, cyfluthrin, and tebupirimiphos, while body weight was significantly different for animals treated with atrazine and tebupirimphos.

According to the report, all the mixtures (0.1 ppb) resulted in significantly smaller size at metamorphosis ($p<0.05$), and mixtures also affected body weight ($p=0.01$), with the mixture of atrazine plus S-metolachlor showing the greatest effect.

Animal size (SVL) at metamorphosis was positively correlated with time to complete metamorphosis for controls. All of the single chemical treatments showed similar positive significant correlations except the treatment with metalaxyl where the correlation, while positive, was not significant. Larvae exposed to atrazine, cyhalothrin, or propioconizole exhibited negative correlations; however, these were not statistically significant. None of the pesticide mixtures resulted in statistically significant correlations except for the 0.1 ppb Bicep II Magnum exposure. The authors noted that for atrazine plus metolachlor, the correlation was negative but nonsignificant.

Body weight of leopard frogs at metamorphosis showed a significant ($p<0.002$) positive correlation with time to complete metamorphosis in controls and with animals treated with alachlor, cyfluthrin, S-metolachlor, metalaxyl, niclosulfuron, and tebupirimiphos individually. Nicosulfuron, atrazine, and cyhalothrin showed positive but not statistically significant correlations.

According to the authors, gonadal development of the study controls was delayed. Testicular oogenesis was not observed in any of the pesticide-treated animals.

Exposure of animals to the nine-chemical mixture was associated with meningitis, otitis interna, and septicemia from gram-negative, water-borne bacteria *Chryseobacterium meninosepticum*.

The incidence of disease in the 59 animals surviving to metamorphosis in the 0.1 ppb chemical mixture group was 70%. The bacterium was successfully cultured from the animals in the other treatment groups even though they did not succumb to disease. Exposure to atrazine and S-metolachlor resulted in damage to the thymus as measured by thymic plaques; this effect, though, was not seen in any of the single pesticide exposure groups. The frequency of plaques increased with animals treated with the mixtures (Bicep II Magnum), followed by larvae treated with the nine-compound mixture (0.1 ppb); however, larvae treated with atrazine plus S metolachlor (0.1 and 10 ppb) were not sampled.

Adult males treated with the nine-pesticide mixture exhibited a significant ($p<0.05$) 4-fold increase in plasma corticosterone levels relative to controls.

The authors conclude that retardation of growth and development was more severe when atrazine was combined with other chemicals such as S-metolachlor. The nine-pesticide mixture had the most severe impact with a significant delay in time to initiate and complete metamorphosis, and this mixture negated or reversed the typically positive correlation between time to metamorphosis and size of metamorphosis. According to the authors, the nine-pesticide mixture induced damage to the thymus, resulting in immunosuppression and contraction of flavobacterial meningitis. This result is attributed to increased plasma levels of the "stress hormone" corticosterone.

Analysis

This study has a number of deficiencies. The light cycle for the study with Northern leopard frogs is not stated, and water quality measurements are not reported. The limit of detection and the limit of quantitation are not stated for the corticosterone radioimmunoassay. The use of plastic mouse cages could result in the leaching of plasticizers, such as phthalates,

that could in turn react with the test chemical and potentially affect the study. Animals were fed a commercial diet (Purina® Rabbit Chow); however, the study does not report whether any analyses were conducted for pesticides and/or phytoestrogens in the food.

Although the statistical analysis section indicates that the frequency of disease transmission and mortality were analyzed, the methods section does not describe the frequency of observations for determining mortality nor does it describe how the frequency of disease transmission was determined

Because the limit of detection for the pesticides (0.1 ppb) was equivalent to the lowest treatment rate, it is uncertain whether the chemical analysis would be capable of confirming exposure concentrations. Measured concentrations are reported for the 0.1 ppb treatment; however, no values are reported for the 10 ppb treatment. Although the report states that no pesticides were detected in the negative control, no information is provided on whether pesticides were detected in the solvent control. Based on measured concentrations, though, exposures deviated by as much as 220% of nominal at the lowest treatment concentration for S-metolachlor.

According to the report, mortality among animals exposed to a single chemical ranged between 0 and 7.8%. Based on the data presented in Table 1 of the report, all of the treatments had a sample size of less than 90 and mortality ranged from 4.4 – 7.8%.

One of the replicates for the metalaxyl group was dropped after a single day because of high mortality following failure of the aeration system. The rapid mortality suggests that loading rates in the tanks were high and that water quality was relatively poor unless supplemental aeration was provided.

Although the report states that pesticide mixtures at 0.1 ppb had significantly longer times to initiate metamorphosis and complete metamorphosis, Figure 2 of the report (**Figure 5** below depicts Figure 2 of the report) indicates that time to initiate metamorphosis was only statistically different for animals treated with atrazine plus S-metolachlor at 10 ppb and for the complete mixture of all pesticides at 0.1 ppb. Again, based on Figure 2 of the report, time to complete metamorphosis was only statistically different for animals exposed to the complete mixture at 0.1 ppb. Although the figure indicates that an asterisk marks statistically significant differences, none of the bars are marked. It is assumed that the authors intended the legend to

read that bars marked with the same letter are not statistically different. Based on the data in this figure, it is unclear how the authors reached the conclusion regarding significantly longer times to initiate metamorphosis for mixtures at 0.1 ppb.

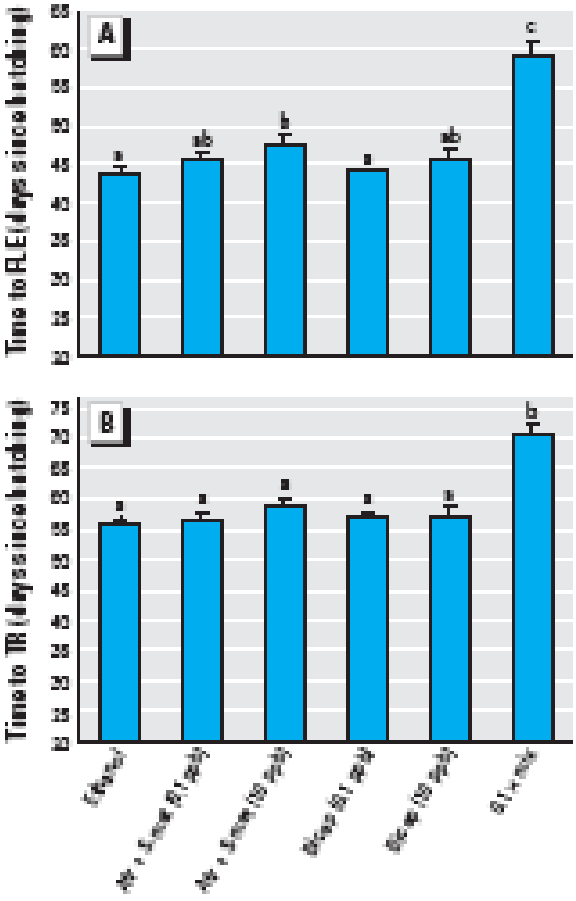

Figure 2. Effect of pesticide mixtures on time to initiate metamorphosis (FLE; *A*) and time to complete metamorphosis (TR; *B*). Abbreviations: Atr, atrazine; Bicep, Bicep II Magnum; S-Met, *S*-metolachlor; Mix, nine-chemical mixture (0.1 ppb each pesticide). Letters above bars indicate statistical groupings. Error bars show SEM.
*Statistically significant groups (ANOVA, $p < 0.05$).

Figure 5. Reproduction of Figure 2 from Hayes *et al.* (2006b) depicting time to initiate (top graph) and complete metamorphosis (bottom graph) for *R. pipiens* exposed to pesticides (Ethanol, Atrazine + S-metolachlor (0.1 ppb), Atrazine + S-metolachlor (10 ppb), Bicep (0.1 ppb), Bicep (10 ppb) and 0.1 ppb mix of all pesticides) mixtures at 0.1 and 10 ppb).

The report states that all the mixtures (0.1 ppb) resulted in significantly smaller size at metamorphosis ($p<0.05$), and that mixtures also affected body weight ($p=0.01$), with the mixture of atrazine plus S-metolachlor showing the greatest effect. However, according to Figure 3 of the

report and assuming that the letters above the bars represent the results of multiple means tests (see **Figure 6** below depicting Figure 3 of the study), SVL was only statistically different for animals exposed to atrazine plus S-metolachlor combination and the pesticide mixture, and body weight was not statistically different across any of the treatments.

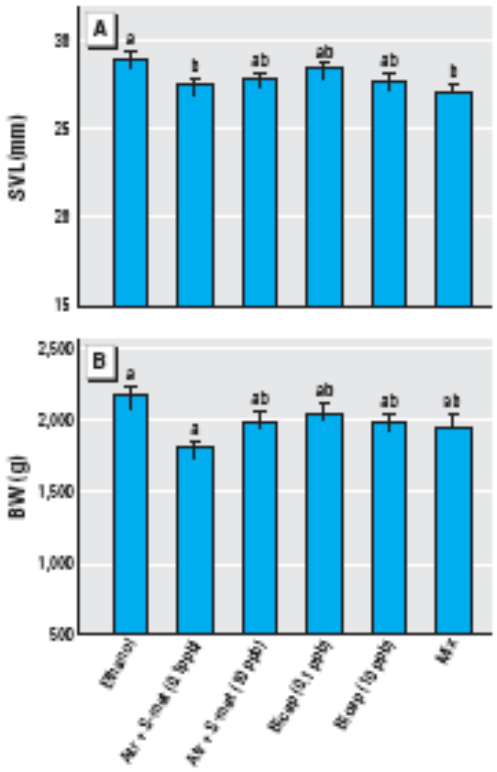

Figure 4. Effect of pesticide mixtures on SVL (*A*) and BW (*B*). Abbreviations: Atr, atrazine; Bicep, Bicep II Magnum; *S*-met, *S*-metolachlor; Mix, nine-chemical mixture (0.1 ppb each pesticide). Letters above bars show statistical groupings. Error bars show SEM.

Figure 6. Figure 4 of the Hayes *et al.* (2006b) study depicting snout-vent length and body weight at metamorphosis for northern leopard frogs exposued to pesticide (Ethanol, Atrazine + S-metolachlor (0.1 ppb), Atrazine + S-metolachlor (10 ppb), Bicep (0.1 ppb), Bicep (10 ppb) and 0.1 ppb mix of all pesticides)

The report discusses the extent to which size (SVL) was correlated with time to metamorphosis. For controls and several of the pesticides, there was a significant positive correlation between the two variables. Because no information is presented on the correlation coefficient, though, it is difficult to gauge the extent to which the correlations were predictive. Table 2 of the report (depicted in **Figure 7** below) provides this information for body weight and suggests that even for the controls where the correlation was positive, it was not particularly

predictive with an r=0.41. The rationale for using statistically insignificant relationships to document potential chemical effects is not provided. The statistically significant correlations for SVL depicted in the report figures suggest that the correlations may have been strongly influenced by relatively few data points and that the majority of points were clustered in no apparent pattern.

Table 2. Statistics for correlational analysis of time to complete TR and BW.

Treatment	*n*	*r*	Chi-squared	*df*	*p*-Value	Figure[a]
Ethanol	86	+0.41	14.61	1	0.000	6A
Alachlor	86	+0.46	16.92	1	0.000	6B
Atrazine	86	+0.07	3.061	1	0.080	6J
Cyfluthrin	86	+0.34	10.03	1	0.002	6C
λ-Cyhalothrin	84	+0.13	1.376	1	0.241	6I
S-Metolachlor	83	+0.47	20.15	1	0.000	6F
Metalaxyl	56	+0.40	9.038	1	0.003	6G
Nicosulfuron	85	+0.49	20.99	1	0.000	6D
Propiconizole	86	+0.20	3.130	1	0.077	6H
Tebupirimphos	85	+0.56	32.77	1	0.000	6E
0.1 ppb atrazine + *S*-metolachlor	89	−0.05	0.251	1	0.616	6K
10 ppb atrazine + *S*-metolachlor	85	−0.01	0.000	1	0.960	6L
0.1 ppb Bicep	81	+0.51	20.69	1	0.000	6M
10 ppb Bicep	84	+0.33	8.143	1	0.004	6N
0.1 ppb mix	59	−0.32	4.164	1	0.041	6O

All treatments were at 0.1 ppb except where indicated for mixtures as described in "Materials and Methods." Bicep II Magnum (Bicep) was administered to provide 0.1 ppb atrazine. The nine-compound mixture (mix) was administered to provide 0.1 ppb of all nine pesticides. Sample size (*n*) represents the number of animals surviving to metamorphosis (out of the original 90; 30 animals in each of three replicates). [a]Indicates the figure number in this article where data are depicted.

Figure 7. Depiction of Table 2 from the Hayes *et al*. (2006b) paper providing statistics for correlation analysis of time to complete metamorphosis and size (snout-to-vent length).

While exposure of Northern leopard frog larvae occurred in aerated exposure solutions, exposure tanks containing adult *X. laevis* were not aerated. Because the clawed frogs are air breathers, the difference between the exposure conditions may have affected pesticide concentrations.

Cardiac puncture on unanesthetized adult animals could also affect blood corticosterone levels. The number of times *Xenopus* males were sampled by cardiac puncture is not stated. Plasma corticosterone levels were approximately 1 ng/ml in the solvent control versus 3 ng/ml in the nine-chemical-treated animals. However, these are relatively low concentrations of corticosterone, and without documentation of the immunoassay's limits of detection and quantitation, it is difficult to put the results into perspective.

Testicular oogenesis was not observed in any of the treated animals, which is a significant departure from what these authors stated as a well documented effect of atrazine, *i.e*, demasculinization and feminization of male larvae (Hayes *et al*. 2002; Hayes *et al.* 2003). The

authors claim that this effect was not observed in the present study because *R. pipiens* do not complete sexual differentiation of the gonads before metamorphosis. However, the animals in this study had completed metamorphosis, and the phenomenon of testicular oogenesis following exposure to atrazine, reportedly observed by this laboratory in previous studies (Hayes *et al*. 2002; Hayes *et al*. 2003), was not observed in this study.

The study does not describe what methods were used to identify how the animals exposed to the nine-chemical mixture exhibited meningitis, otitis interna, and septicemia from gram-negative water-borne bacteria *Chryseobacterium meninosepticum.* None of the methods used to identify these pathologies were discussed in the methods section.

The authors claim that the reversal in the relationship between time to metamorphosis and size at metamorphosis has also been demonstrated by Carr *et al*. (2003). However, this study did not show a significant cause-effect relationship for atrazine (USEPA 2003). Furthermore, the Carr study was reviewed by the Agency (USEPA 2003) and was determined to have such detrimental culture conditions that amphibian development could not be reasonably gauged in the study.

The authors conclude that retardation of growth and development was more severe when atrazine was combined with other chemicals such as S-metolachlor, and the nine-pesticide mixture had the most severe impact with a significant delay in time to initiate and complete metamorphosis. However, based on the report figures, neither atrazine nor S-metolachlor alone appeared to impact development time, and only the time to initiate metamorphosis was significantly different (longer) than controls for atrazine combined with S-metolachlor at 10 ppb. Neither the combination at 0.1 ppb nor the formulated product tests indicated any significant delay on time to initiate or complete metamorphosis. Therefore, the authors' conclusions regarding the effects of the mixture and its potential effects on survivorship do not appear to be supported by the data depicted in the paper except for the highest concentration of atrazine plus metolachlor.

Furthermore, the authors indicate that smaller size at metamorphosis makes juveniles more vulnerable to predation; however, the only pesticide mixture that resulted in statistically significant effects on size in terms of length (SVL) was atrazine plus metolachlor at the lowest

concentration (0.1 ppb); none of the pesticide mixtures including the 9-chemical mixture resulted in statistically significant effects on body weight of the animals at completion of metamorphosis.

Although the study authors make considerable use of trends in their study, most statistical tests are intended to determine whether a measured effect would likely occur from chance alone or whether there is a difference between treatments based on a given level of probability. For many of the endpoints discussed in this study, there was no statistically significant difference between treatments and controls even though the authors claimed there were effects. The authors attempted to draw conclusions from statistical tests where there were no statistical differences. Finally, although the authors have asserted previously that atrazine exposure feminizes and demasculinizes amphibians, this study did not show any effects of atrazine on amphibian gonadal development. The authors attribute this result to biological variability across experiments, a conclusion that conflicts with their previous assertion of a consistent response from chemical exposure to atrazine (Hayes *et al*. 2002; Hayes *et al*. 2003).

Additionally, the study concludes that the nine-pesticide mixture induced damage to the thymus resulting in immunosuppression and contraction of flavobacterial meningitis; however, the study does not provide any evidence that plaques on the thyroid are in any way correlated with immunosuppression, which in turn leads to flavobacterial meningitis. Given the range of bacteria, fungi, and viruses that must exist in and around the animals in this study, it is unclear why the study implies that the chemical treatment would lead to this specific disease. Finally, concluding that the high incidence of bacterial disease in the *R. pipiens* larvae may be caused by increases in plasma corticosterone levels observed in adult *Xenopus laevis* does not appear to be supported by the data since corticosterone levels were not measured in the *R. pipiens* and the flavobacterial disease was not observed in the *Xenopus* adults.

Although the authors report on atrazine alone and in combination with several pesticides, the focus of this White Paper is the effect of atrazine alone. The authors rely on study methods (plastic mouse cages and high loading rates) that were discussed in the previous White Paper (USEPA 2003) as potential sources of variability. Atrazine concentrations reported in the study were at the limit of detection for one of the treatment groups (0.1 ppb) and it is unclear whether exposure concentrations were verified for the highest treatment concentration (10 ppb). Additionally, the authors draw conclusions from relationships that are not statistically significant and appear to misinterpret the statistical significance of some of the data presented in their report

figures. In this study, the authors report no significant effect of atrazine on time to and size at metamorphosis nor does atrazine exposure result in effects on gonadal development in Northern leopard frogs. The absence of an effect on gonadal development in *Rana pipiens* appears to contradict the results of previous studies from this laboratory and represents a major inconsistency. The authors attribute the lack of response in this study to biological variability.

Hecker, M., J.W. Park, M. B. Murphy, P. D. Jones, K. R. Solomon, G. Van Der Kraak, J. A. Carr, E. E. Smith, L. Du Preez, R. J. Kendall, and J. P. Giesy. 2005a. Effects of Atrazine on CYP19 Gene Expression and Aromatase Activity of Testes and on Plasma Sex Steroid Concentrations of Male African Clawed Frogs (*Xenopus laevis*). Toxicological Sciences 86(2): 273 – 280.

Description and Results of this Study

The purpose of this study was to analyze the mechanism of action for atrazine on the induction of aromatase in testes of male adult African clawed frogs, *Xenopus laevis*. Adult, (30 – 50 g) sexually mature male *X. laevis,* obtained from Xenopus Express (Plant City, FL), were acclimated for several weeks in 600-L fiberglass tanks under flow-through conditions at $19.6^{o} \pm 1.3^{o}$C with a 12:12 hr light:dark cycle. Animals were fed frog brittle (Nasco, Fort Atkinson, WI) 3 times per week *ad libitum*.

Frog exposures to atrazine (97.1%; Syngenta Crop Protection) at 0, 1, 25, or 250 μg/L were conducted under static-renewal conditions for 36 days with 50% water changes every 3 days. Frogs were exposed individually in 40-L aquaria containing 10 L of exposure solution; there were 15 replicates per atrazine treatment and 20 replicates for the control group. Concentrations of atrazine were verified by randomly selecting aquaria within each of the treatments every 5 – 7 days; atrazine concentrations were measured using gas chromatography/mass spectroscopy.

After 36 days of exposure, frogs were anesthetized with tricaine methanesulfonate, and blood was collected by cardiac puncture. Plasma was separated by centrifugation (10,000 g for 10 min) and stored at $^{-}80^{o}$C. Wet weight and snout-vent-length were measured. Gonads were removed, measured, weighed and morphological abnormalities noted if present. Each gonad was split into two equal portions and the portions from each gonad were mixed and then frozen in liquid nitrogen. The gonadosomatic index (GSI) was calculated as:

$$GSI=(\text{gonad weight} \div \text{body weight}) \times 100$$

The condition index (CI) was calculated as:

$$CI=(\text{body weight} \div \text{SVL}) \times 100$$

Aromatase activity was measured by use of the tritiated water release assay according to the protocol of Lephart and Simpson (1991). The limit of detection (LOD) for aromatase activity was 1.8 fmol/hr/mg protein. Protein concentrations were determined using the Bradford assay (Bradford 1976), and CYP19gen expression in testes was measured by means of a quantitative reverse transcription real-time polymerase chain reaction (Q-RT-PCR) technique. Plasma sex steroids (estradiol and testosterone) were measured by competitive enzyme-linked immunosorbent assay (ELISA).

According to the study authors, there were no differences in water quality (NO_2, NH_3, dissolved oxygen, pH, hardness or temperature) among tanks or treatments. The average procedural recovery for atrazine was 92±7%; the limit of quantification for atrazine was 0.025 μg/L.

Measured atrazine concentrations averaged 0.8 ± 0.11, 24.6 ± 2.1 and 258 ± 29.1 μg/L (mean ± std error) in the 1, 25, and 250 μg/L treatments, respectively. The study reports that atrazine concentrations were stable in all treatments over the entire duration of the study. Mortality was 5%, 0%, 6.7%, and 13% in the 0, 1, 25, and 250 μg/L groups, respectively. Condition indices in the 25 and 250 μg/L treatments averaged 0.73 ± 0.02% and 0.68 ± 0.03%, respectively.

According to the study, no morphological abnormalities of the testes such as discontinuous gonads or occurrence of ovarian tissue were observed.

For GSI, none of the atrazine treatments were statistically different (p=0.633) from controls; GSI was correlated with CYP10 mRNA copy number in the 1 μg/L treatment group. Neither gonadal aromatase nor gonadal CYP19gen expression were significantly different among any of the treatments. No correlations between aromatase activity and CYP19gen expression were observed except at the highest atrazine treatment concentration of 250 μg/L. In the 250 μg/L treatment group, there was a significant negative correlation between CYP19gen MRNA and aromatase activity (R^2=0.675, p=0.001).

None of the atrazine treatments significantly affected plasma testosterone or estradiol concentrations except for the highest atrazine concentration in which plasma testosterone was significantly (p=0.036) reduced (7.1 ng/ml) relative to controls (15.3 ng/ml). (**Figure 8** below depicts Figure 3 of the study.) There was a concentration-dependent negative relationship between average plasma testosterone concentration and atrazine exposure concentration.

Atrazine treatment had no significant effect on estradiol concentrations. According to the study authors, plasma estradiol concentrations were relatively constant across all treatments and ranged between 3.1 ng/ml in controls to 3.6 ng/ml in the 25 μg atrazine/L treatment. Neither plasma testosterone nor estradiol was correlated with GSI, aromatase activity, CYP19gen expression, or with each other.

The authors concluded that atrazine treatment for 36 days had no effect on testicular size nor did it affect testicular aromatase in male *X. laevis* at either the gene expression level or the enzyme activity level. Atrazine exposure did appear to affect plasma testosterone homeostasis as indicated by a significant reduction in plasma testosterone associated with exposure to atrazine at 250 μg/L. Although this effect has been reported in other studies, the study did not show atrazine-related effects on testosterone concentrations or aromatase activity and does not support the hypothesis that atrazine exposure up-regulated aromatase. The study, however, did demonstrate a significant negative correlation between CYP19gen expression and testicular aromatase activity in frogs exposed to 250 μg/L compared to controls, and the authors speculated on the potential effects on aromatase at higher atrazine concentrations. They also speculated that atrazine may act on a more general mechanism such as an increase in metabolic activity rather than on specifically targeting estrogen synthesis. Finally, the authors noted that the concentration of atrazine which showed an effect on testosterone homeostasis was roughly an order of magnitude higher than peak surface water monitoring concentrations.

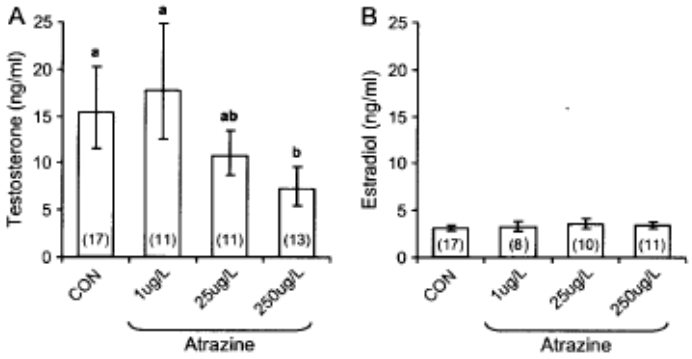

FIG. 3. Geometric mean plasma testosterone (A) and estradiol (B) concentrations detected in adult male *X. laevis* exposed to control water (CON) and atrazine. Error bars = standard error. Letters (a–b) signify tests for statistically significant differences at $p < 0.05$ between treatments. Numbers in parentheses indicate number of individuals per treatment.

Figure 8. Depiction of Figure 3 from Hecker *et al*. 2005a showing geometric mean plasma testosterone (A) and estradiol (B) concentrations by treatment.

Analysis

The study does not contain information for several experimental parameters. Composition of the exposure aquaria is not stated in the methods section. Commercial diet (Nasco™ frog brittle) was used; however, the study does not report whether it was analyzed for pesticides and/or phytoestrogens.

According to the methods section, body weights were recorded after blood had been collected from the frogs via cardiac puncture. The methods section does not state the volume of blood sampled from each of the frogs, but it is likely that the sampling affected the total body weight of the animals. Presumably, body weights were recorded after the animals had been blotted dry. Potential effects of cardiac puncture on weight could in turn affect any estimates dependent on body weight such as GSI and CI.

The methods section states that the gonads were split in half, and portions from the right and left testis were combined; however, the protocol later states that for the aromatase assay, 0.5 g (one testis) was homogenized. It is unclear whether the protocol intended to state that the sample size was equivalent to the weight of one testis.

Although measured atrazine concentrations were relatively close to nominal, the methods section does not describe when treatment solution concentrations were verified, *i.e*., whether samples were taken prior to or after renewal.

The average condition index was only presented for the 25 and 250 μg/L treatments. Although the report states that the condition index was similar throughout all treatments, no statistical analysis was presented to substantiate whether the mean condition indices reported for the two highest atrazine treatment concentrations were significantly different.

The study reports that no gonadal abnormalities were observed such as the occurrence of ovarian tissue in the testes; however, it is unlikely that, based on gross morphology, the authors could detect the presence of ovarian tissue in the gonads.

Although the limit of detection (LOD) for aromatase activity was 1.8 fmol/hr/mg protein, the limit of quantification for aromatase activity is not stated. Neither the limit of detection nor the limit of quantification is provided for the plasma steroid (ELISA) analyses.

Figure 9 depicts geometric plasma testosterone and estradiol concentrations across treatments. It is unclear why the sample size is different for testosterone versus estradiol since the samples were presumably collected from the same animals. It is also unclear why the sample numbers do not reflect the number of animals reported to have survived in each of the treatments unless blood samples could not be obtained from all of the animals.

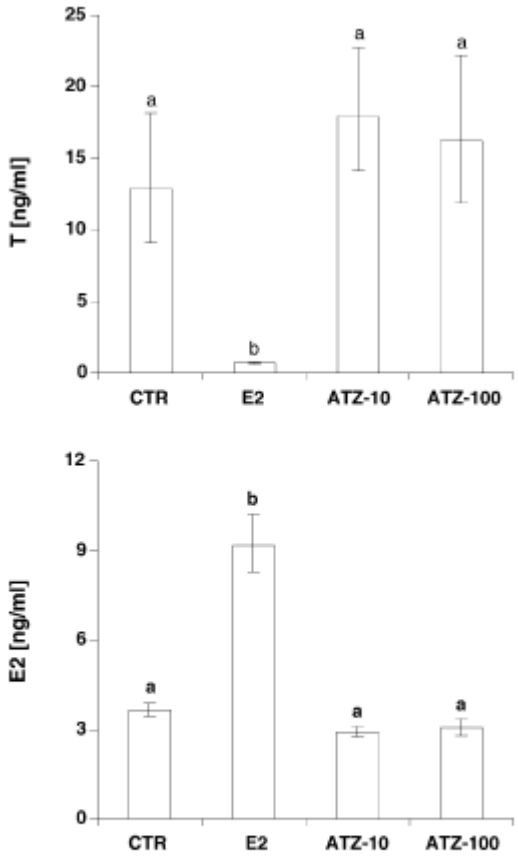

Fig. 5. Geometric mean plasma testosterone (T) (top) and estradiol (E2) (bottom) concentrations detected in adult male *X. laevis* exposed to control water (CTR), 10 or 100 µg/L atrazine (ATZ-10, ATZ-100), or 0.1 µg/L estradiol (E2). $n = 6–8$ frogs per tank, 18–19 frogs per treatment. Error bars = standard error. Letters (a, b) signify tests for statistically significant differences at $p < 0.05$ between treatments.

Figure 9. Plasma testosterone (A) and estradiol (B) concentrations across treatments (Control, Estradiol, Atrazine 10 ppb and Atrazine 100 ppb) as represented in Figure 5 of Hecker *et al.* 2005a.

Although the authors note that [geometric] mean plasma testosterone was negatively correlated with atrazine concentration, they do not provide any statistics on their analyses. Plasma testosterone concentrations in males treated with atrazine at 25 and 250 μg/L appear to be reduced relative to controls; however, only the 250 μg/L treatment group was demonstrated to be significantly different. High variability in plasma testosterone concentrations may have reduced the power of the study to detect potential treatment effects.

In general, the study authors have attempted to address animal husbandry and exposure conditions that have been sources of variability in the studies reviewed for the 2003 SAP; however, it is unclear what material was used in the construction of exposure chambers, *e.g.*, glass or fiberglass, and whether the animal diet was evaluated for potential contamination with

atrazine and/or phytoestrogens. The conclusions drawn by the study authors appear to be consistent with the information presented in their paper. The data indicate that atrazine exposure did not affect testicular aromatase in male *X. laevis* at either the gene expression level or the enzyme activity level. Additionally, no morphological abnormalities of the testes were observed. Plasma testosterone was significantly reduced relative to controls following exposure to atrazine at 250 μg/L; however, there was no significant dose-response to atrazine at the concentrations tested. The concentration of atrazine and the exposure duration required to reduce plasma testosterone concentrations are high compared to what may be encountered in the environment. Based on the results of this study, the reduction in plasma testosterone observed following 36 days of exposure to atrazine at 250 μg/L did not appear to be a result of an up-regulation of testicular aromatase activity.

Hecker, M., W. J. Kim, J. W. Park, M. B. Murphy, D. Villeneuve, K. K. Coady, P. D. Jones, K. R. Solomon, G. Van Der Kraak, J. A. Carr, E. E. Smith, L. Du Preez, R. J. Kendall and J. P. Giesy. 2005b. Plasma Concentrations of Estradiol and Testosterone, Gonadal Aromatase Activity and Ultrastructure of the Testis in *Xenopus laevis* Exposed to Estradiol or Atrazine. *Aquatic Toxicology* 72: 383 – 396.

Design and Results of Study

The purpose of this study was to identify possible effects of atrazine on testicular ultrastructure and to establish typical effects of exposure to estradiol on testicular ultrastructure in adult *X. laevis* as a sub-cellular marker for estrogen exposure in amphibians. For the test solution, atrazine (97.1%; Syngenta Crop Protection) was dissolved in ultra violet light-treated laboratory water. Ethanol (100%; AAPER Alcohol) was used as a co-solvent for the 17β-estradiol (98%; Sigma Chemical Co.) control and the final ethanol concentration in exposure solutions was 0.005%. An ethanol solvent control was not included. Adult male *X. laevis* (30 – 50 g) were purchased from Xenopus Express (Plant City, FL). Animals were acclimated for several weeks in 600-L fiber glass tanks under flow-through conditions at 18.5 – 23.5^{o}C and maintained under a 12:12 hr light:dark cycle. Animals were fed Nasco® frog brittle (Nasco, Fort Atkinson, WI) three times per week *ad libitum*.

Animals were exposed in 600-L fiberglass tanks filled with 120 L test solution. Frogs were exposed to 0, 10, 100 µg atrazine/L, or 0.1 µg estradiol/L, and the study was conducted in triplicate under static-renewal conditions with 50% water renewals every 3 days. Feeding, temperature, and photoperiod were similar to the acclimation period. Initially, 14 males (42 ± 6.6 g) per tank were used, representing a loading rate of 4.9 g frog/L. Seven frogs were sampled

from each exposure tank on exposure day 49. Animals were anesthetized with tricaine methanesulfonate, and blood was collected via cardiac puncture. Plasma was separated by centrifugation at 10,000 g for 10 minutes. Wet weight and snout-vent length (SVL) were measured, and the gonadosomatic index (GSI) was calculated as:

GSI= (gonad weight ÷ body weight) x 100

Two animals from each treatment were used for ultrastructural analysis of testes using transmission electron microscopy. Phenotypic sex was determined by size and external sexual dimorphic characteristics and by inspection of gonads.

Water samples were collected from each of the three replicates before and slightly after each solution renewal. Samples were analyzed using enzyme-linked immunosorbant assay (ELISA; Envirogard® Triazine Plate Kits; Strategic Diagnostics, Newark, DE); the limit of detection was 0.02 µg/L, and the limit of quantification was 0.22 µg/L.

Plasma estradiol and testosterone concentrations were determined by ELISA. Estradiol concentrations in the positive control were measured by ELISA using COSTAR high binding plates. Working ranges of these assays were 0.78 – 800 pg/well for both estradiol and testosterone.

Gonadal aromatase activity was measured by use of the tritiated water release assay according to the protocol of Lephart and Simpson (1991); the limit of detection (LOD) for aromatase activity was 0.22 fmol/hr/mg protein.

Mean-measured concentrations for atrazine were 11.6 ± 1.68 µg/L and 107 ± 20.7 µg/L in the 10 and 100 µg/L atrazine treatments, respectively. Atrazine in the negative and estradiol controls was less than 0.074 µg/L. The median difference between before- and after-renewal atrazine concentrations was not significantly different from 0 for any of the treatments ($p \geq 0.06$).

A single mortality was reported in the estradiol group. In addition, a variety of ultrastructural changes were observed in the testes of estradiol-treated animals relative to controls: prominent nucleoli and no chromosome condensation in primary spermatocytes; significantly fewer spermatids and sperm; decreased average number of sperm heads (18%)

within a defined area; incomplete condensation of sperm nuclei; swellings and irregularities of the nuclear envelope and less developed endoplasmic reticulum of Leydig cells; increased number of vacuoles and inclusion bodies in spermatids and sperm heads; less differentiated Leydig cells; and increased number of vacuoles and lipid droplets in the cytoplasm of Lydig cells.

According to the authors, there were no ultrastructural differences in testes between control and atrazine-treated frogs. Other cell organelles and constituents of developing germ cells and sperm were similar to those of the control group. Numbers of sperm heads (25.4 ± 1.1 and 24.7 ± 1.4) in the 10 and 100 µg/L atrazine treatments, respectively, were not significantly ($p \geq 0.26$) different from controls (27.3 ± 1.7) nor from the estradiol (22.3 ± 1.5) treatment group. **Figure 10** below depicts Figure 2 of the paper and shows the number of sperm per area across treatments.

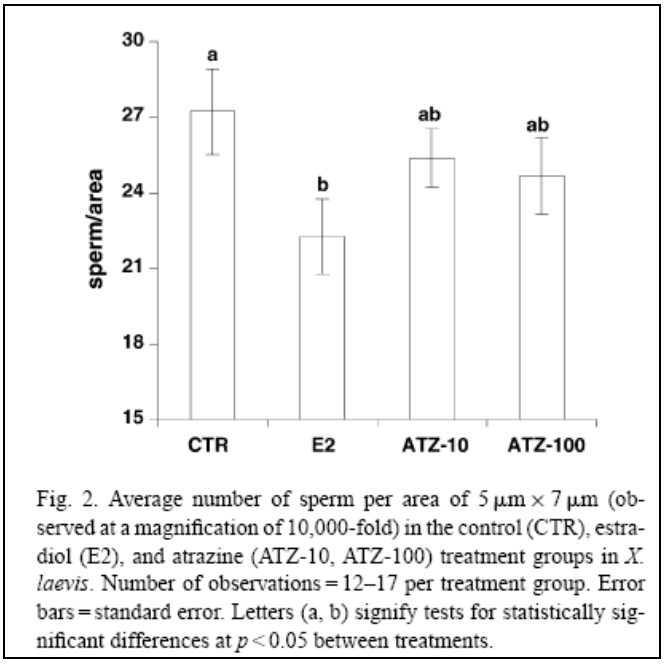

Fig. 2. Average number of sperm per area of 5 µm × 7 µm (observed at a magnification of 10,000-fold) in the control (CTR), estradiol (E2), and atrazine (ATZ-10, ATZ-100) treatment groups in *X. laevis*. Number of observations = 12–17 per treatment group. Error bars = standard error. Letters (a, b) signify tests for statistically significant differences at $p < 0.05$ between treatments.

Figure 10. Number of sperm per area across controls represented by Figure 3 of Hecker *et al.* (2005b).

In this study, exposure to atrazine did not significantly affect either plasma estradiol or testosterone concentrations. **Figure 11** below depicts Figure 5 of the paper and shows plasma testosterone (**A**) and estradiol (**B**) concentrations by treatment. Estradiol treatment significantly ($p<0.001$) reduced plasma testosterone concentrations compared to controls; however, the authors noted that high variability limited the power of the study to detect potential treatment effects.

Although neither atrazine nor estradiol affected testicular aromatase activity, there was no detectable aromatase activity in any of the testicular tissues assayed. Positive controls with ovarian tissue indicated that the aromatase assay was functional.

Frogs from both atrazine treatments had significantly ($p \leq 0.007$) greater median GSI than estradiol-treated frogs but were not significantly different than controls ($p=0.145$). However, mean GSI for animals treated with atrazine at 10 µg/L was significantly ($p=0.046$) greater than controls (**Figure 12**).

Since aromatase activity in estradiol and atrazine-treated animals was not statistically different from controls, the authors concluded that the increase in plasma estradiol in estradiol-treated animals is not a function of increased endogenous estradiol production, but is likely due to other factors such as accumulation of estradiol from the test solution. The authors also concluded that atrazine exposure did not result in any changes in testicular ultrastructure; the authors speculated that the statistically significant reduction in GSI among animals treated with atrazine at 10 µg/L may be related to reduced food availability.

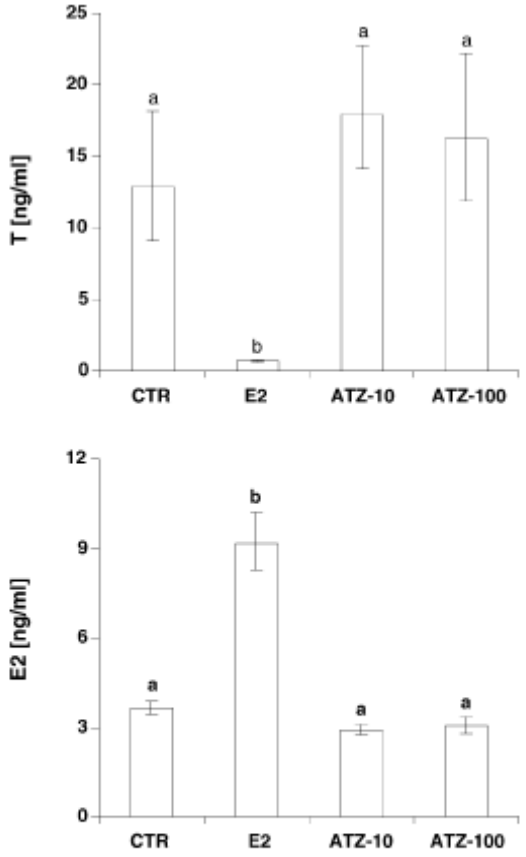

Fig. 5. Geometric mean plasma testosterone (T) (top) and estradiol (E2) (bottom) concentrations detected in adult male *X. laevis* exposed to control water (CTR), 10 or 100 μg/L atrazine (ATZ-10, ATZ-100), or 0.1 μg/L estradiol (E2). $n = 6–8$ frogs per tank, 18–19 frogs per treatment. Error bars = standard error. Letters (a, b) signify tests for statistically significant differences at $p < 0.05$ between treatments.

Figure 11. Plasma testosterone (A) and estradiol (B) concentrations across treatments as represented in Figure 5 of Hecker *et al.* 2005b.

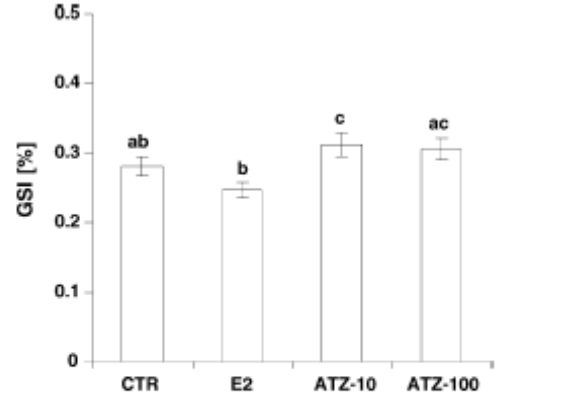

Fig. 6. Mean gonadal somatic index (GSI) for adult male *X. laevis* exposed to 10 or 100 µg/L atrazine (ATZ-10, ATZ-100), control water (CTR), or 0.1 µg/L estradiol (E2). $n = 20–21$. Error bars = standard error. Letters (a–c) signify tests for statistically significant differences at $p < 0.05$ between treatments.

Figure 12. Mean GSI across treatments as represented in Figure 6 of Hecker *et al.* 2005b.

Analysis

This study had a number of deficiencies. The study was conducted in a fiberglass tank, whereas EPA recommends the use of glass, stainless steel or teflon-lined exposure tanks. Water quality conditions were not reported for the exposure tanks in terms of nitrate, nitrite, and ammonia levels.

Loading rates (4.9 g/L) were higher than the ASTM-recommended 1 g/L. This deficiency combined with *ad libitum* feeding and only partial (50%) water renewals every third day may have been a confounding factor in the ability of the study to document any effects due to atrazine exposure alone.

An ethanol control was not run with the estradiol. The study authors acknowledge that the potential role of ethanol in the effects associated with estradiol in this study cannot be discounted.

Animals were fed commercial diet; however, no information is presented on whether the feed was analyzed for pesticides and/or phytoestrogens. The methods section does not indicate how protein concentrations were determined; however, previous papers by Hecker *et al.* (2005) have indicated that protein concentrations were determined using the Bradford (1976) assay.

While not statistically significant, the reduced number of sperm heads in testes from atrazine-treated animals appears to be the only notable, albeit statistically insignificant, difference between atrazine and control animals. Although the number of spermatids was significantly lower in estradiol-treated animals compared to controls, there was no statistical difference between estradiol-treated and atrazine-treated animals; however, the fact remains that atrazine-treated animals were not significantly different from controls either.

It is unclear why statistical comparisons are made using both the median and mean measures of central tendency. The lack of statistical significance using median GSI versus the statistically significant difference between atrazine (10 µg/L) and controls for mean GSI (**Figure 12**) suggests that the data were not normally distributed, and it is unclear whether similar manipulations would have revealed statistical differences for other measurement endpoints in this study.

As in the previous study, the authors use sexually mature frogs and thus this study cannot address the issue of whether atrazine affects time to or size at metamorphosis. Animal husbandry in terms of loading rates and water changes were not consistent with what is recommended by ASTM and atrazine concentrations were not reported as measured in the diet. In general, this study suggests that atrazine treatments only appeared to affect GSI; however, this effect was not dose-dependent. The study conclusions appear to be consistent with the data presented in the study; however, the repeated reference to the statistical power of this study to discriminate effects suggests that high variability may have limited the ability of the study to discriminate treatment effects. Additionally, the limited sensitivity of the aromatase assay and its inability to detect quantifiable activity in any of the samples [other than ovarian tissues] may have precluded testing whether atrazine affects aromatase activity in males.

Orton, F., J. A. Carr and R. D. Handy. 2006. Effects of Nitrate and Atrazine on Larval Development and Sexual Differentiation in the Northern Leopard Frog *Rana pipiens*. Environmental Toxicology and Chemistry 25(1): 65 – 71.

Description and Results of Study

The purpose of this study was to expose early life stages of Northern leopard frogs (*Rana pipiens*) to environmentally realistic combinations of nitrate and atrazine to quantify morphological changes through measurements of growth, development, and reproductive

anatomy. In this study, laboratory-reared *R. pipiens* were obtained from Carolina Biologicals (Burlington, NC) and maintained in aged (dechlorinated) tap water (Lubbock, TX). Tadpoles were acclimated for roughly two weeks. Each experimental unit consisted of a tank containing 8 L of water and 20 4-wk old tadpoles. Animals were fed Purina® Rabbit chow. Initially, water within each of the tanks was renewed by 50% every 48 hours. At metamorphosis, climbing racks were placed on each of the tanks to prevent the juveniles from drowning. Tanks were kept on a 14:10 light:dark cycle at 21.8±0.03°C (mean ± std err) and the position of the tanks was rotated during the study to minimize confounding tank location effects. Tadpoles were exposed from Taylor-Kollros stage 2/3 until stage 23/24 (time 10 – 12 weeks) when they were euthanized. Four experimental treatments were conducted: control (aged tap water alone), nitrate (10 mg/L in aged tap water), atrazine (10 µg/L in aged tap water), and 10 mg nitrate (99% purity) plus 10 µg atrazine (98.2% purity)/L combined in aged tap water. Each treatment was replicated 4 times with a static renewal and 75% water replacement every 2 days. Observations for mortality, abnormal swimming, edema, and forelimb emergence were recorded every 48 hours. At stage 23/24 (NF Stage 66), metamorphs were removed and euthanized using methyl tricaine sulfonate, the animal weight recorded, and each frog was then fixed in Bouin's solution. After fixation, hind-limb length, snout-vent length, total length, and sex were recorded. Histological analysis was then conducted on the gonads of each animal.

Verification of atrazine concentration was made using a polyclonal ELISA; nitrate concentrations were measured using Test "N" Tube chromatographic acid method (Hach). Nitrate and atrazine concentrations were measured in stock solutions and in the tanks at the beginning, middle, and end of the exposure.

Histological analysis was performed on the gonad from each unknown sex individual and on randomly selected anatomical males and females from each tank. Representative sections of the dorsal, middle, and ventral parts of each gonad were examined. The left ovary was examined in all females, and in males, both testes were examined.

Water quality measurements indicated mean (±std error) dissolved oxygen (8.08±0.01 mg/L), pH (8.08±0.007), temperature (21.8±0.03°C), total ammonia (4.37±0.11 mg/L), and un-ionized ammonia (0.238±0.006 mg/L).

Overall measurements for nitrate in each of the treatments were: control (0.64±0.099 mg/L; mean ± std error), nitrate (8.33 ± 0.44 mg/L), atrazine (0.61 ± 0.08 mg/L) and combined treatment (7.86 ± 0.5 mg/L). Measured concentrations of atrazine were: control (<0.1 µg/L), nitrate (<0.1 µg/L) atrazine (15 ± 1.5 µg/L), and combined treatment (16 ± 2.2 µg/L).

Cumulative mortalities per replicate were significantly ($p<0.05$) lower in the nitrate (1.25±0.25), atrazine (0.25±0.96), and combined (2.55±0.75) treatments than in controls (10+1.68). The incidence of larvae with bent tails (2 – 4/tank) or edema (0 – 2/tank) was low and was not significantly different between treatments. There were no significant differences ($p>0.05$) in body weight (roughly 1.2 g), SVL (roughly 20 mm) and hind-limb length (roughly 29 mm) between treatments. Time to initiate metamorphosis (forelimb emergence) in controls (26 days) was not significantly different than 28 to 29 days among treated animals.

Histology was reported to have provided a more accurate estimate of sex compared to gross morphology of the gonad. Sex ratio was significantly ($p=0.0065$) different in treated groups compared to controls. The proportion of females was higher in all treatments compared to controls (**Table 21**). The proportion of males decreased in the combined treatment. The frequency of intersex was highest in the controls; however, there was no statistical difference between treated and control animals.

Table 21. Sex ratios based on histological analysis of gonads.

Treatment	Male	Female	Intersex
Control	46.5±6.91	41±7.12	12.5±8.8
10 mg/L nitrate	51.15±7.9	44.6±6.32	4.25±2.8
10 µg/L atrazine	50±7.48	47±7.4	3±1.71
10 mg nitrate + 10 µg atrazine/L	33.6±4.04	66.4±4.0	0

Histology of the testes revealed no significant difference in the total number of germ cells; however, nitrate and atrazine treatments displayed significantly ($p=0.02$) higher percentage of spermatgonia, and all treatments displayed significantly lower percentage of spermatocytes ($p=0.001$) and significantly ($p=0.001$) higher percentage of spermatids compared to controls. There was no correlation between the pattern of testicular morphology and the incidence of mortality.

According to the authors, neither nitrate nor atrazine alone caused significant ($p<0.01$) increases in the follicular size of ovaries compared to controls, and the combined treatment showed significantly ($p<0.01$) increased size of mature follicles compared to controls. In addition, there was no correlation between the incidence of mortality and follicle size.

Testicular oocytes were used to identify individuals as intersex. The cumulative number of testicular oocytes was higher in nitrate (22) and atrazine (19) treatments relative to controls (13), but no testicular oocytes were observed in the combined treatment. Testicular oocytes were significantly ($p<0.01$) larger (1.5-fold) in the nitrate-only treatment (76.8±4.6 μm) compared to the controls (49.6±4.4 μm), while in the atrazine-only treatment, they were significantly smaller (3.58±1.2 μm) than the controls.

The authors conclude that growth and larval development were not affected by the treatments, but effects on gonadal development were observed, including decreased percentage of spermatocytes and an increase in the percentage of spermatids in the testes of nitrate- and atrazine-treated groups compared to controls. In females, follicle size was increased in both nitrate- and atrazine-treated groups compared to controls. The authors speculate that atrazine or nitrate could accelerate the gonadal maturation process. Atrazine and nitrate combined resulted in a higher percentage of female frogs. The authors state that demasculinization of existing male gonads seems unlikely since the production of spermatids did not decline in any treatments. The relative increase in the proportion of spermatids and decrease in the proportion of spermatocytes in treated groups was considered diagnostic of accelerated maturation of sperm. Similarly, the authors conclude that follicular maturation was accelerated in the separate atrazine and nitrate treatments but only had limited effect in the combined treatments.

Analysis

This study is included in the analysis since it contains information on the potential effects of atrazine on amphibian gonadal development. The loading rate used in the study (20 tadpoles/8 L) exceeds the ASTM-recommended rate of 1 tadpole/L. Although not exactly clear in the results section, individual tank (replicate) mortality in the controls averaged 10 frogs and indicates a mortality rate of 50%. Mortality rates in the treated groups were roughly 5%. The high mortality in the controls confounds this study and renders it of questionable utility in distinguishing any effects of development since the control animals were apparently struggling to survive. While the

authors state that other studies with *R. pipiens* have exhibited mortality ranging from 37 – 58%, these high mortality rates indicate that in those studies, culture conditions were not ideal either. While water quality parameters appear to have been reasonable, it is unclear why such differential mortality occurred in the controls.

Animals were fed commercial diet (Purina Rabbit Chow®); however, no information is presented on whether the feed was analyzed for pesticides and/or phytoestrogens.

The average size of testicular oocytes reported for atrazine-treated frogs (3.58±1.2 µm) is substantially lower than the 0.7-fold value reported in the text. Presumably, the value is a typographical error and should read 35.8±1.2 µm.

The relatively high, albeit statistically insignificant, incidence of intersex in control animals is unclear since the treated groups appear to have a much lower incidence. The changes in sex ratios observed in the treated groups are difficult to interpret since only one concentration of each treatment was tested. It isn't possible from the study design to determine whether a dose-dependent effect exists. Additionally, the sex ratios in both the atrazine-treated and nitrate-treated animals appear to approximate the expected 50:50 ratio than what was observed in the controls.

The study measured atrazine concentration in the exposure solutions; however, it is unclear whether the feed used in the study was analyzed for atrazine and/or phytoestrogens. Major sources of uncertainty include loading rates that exceeded the recommended levels and high percent mortality in the controls. Additionally, there was a relatively high incidence of intersex observed in the controls. The ability of the study to detect treatment effects is limited by these deficiencies and thus the objective of the study is compromised.

Sullivan, K. B., and K. M. Spence. 2003. Effects of sublethal concentrations of atrazine and nitrate on metamorphosis of the African clawed frog. Environmental Toxicology and Chemistry 22(3): 627 – 635.

Description and Results of Study

The purpose of this study was to examine the potential interactive effects of nitrate and atrazine on the metamorphosis of the African clawed frog (*Xenopus laevis)*. In this study, adult

X. laevis were obtained from Xenopus I (Dexter, MI) and were maintained in laboratory dechlorinated tap water; three adult pairs were induced to spawn. Six tadpoles, age 9 to 11 days post-hatch (Nieuwkoop Faber Stage 46 – 48), were added to 600-mL glass beakers containing 500 mL of test solution; there were 5 replicates per treatment. Three distinct studies were conducted: exposure to atrazine (99% pure) at nominal concentrations of 0, 20, 40, 80, 160, and 320 µg/L through metamorphosis; exposure to nitrate ($NaNO_3$ 99.5% pure) at nominal concentrations of 0, 7, 37, 73, 146, and 292 mg/L through metamorphosis; a factorial design of three nominal concentrations of atrazine (0, 40, and 320 µg/L) and three nominal concentrations of sodium nitrate (0, 37, and 292 mg/L) crossed for a total of nine treatment concentrations. After tadpoles had reached 26 – 33 day old, they were transferred to 1.5 L of medium hard water in 1 quart glass Mason jars for the atrazine and nitrate tests. When tadpoles were 45 – 50 days old, replicate containers were combined into 6 L medium hard water in 10-gal (37.8-L) tanks. Tadpoles in the mixture study were introduced into 2.5 L of medium hard water in 37.8-L aquaria at day 10 of age and after 2 weeks, the water volume was increased to 6 L. When only half the number of larvae were contained in a tank, the volume was reduced to 3 L. All tanks were tilted to increase the depth. Each tank was continuously aerated through use of an air stone. Each of the nine treatment combinations was replicated in 3 aquaria with 30 tadpoles assigned to each tank except for one replicate (320 µg atrazine plus 0 mg nitrate/L), which contained 35 tadpoles. Animals were fed *ad libitum* with commercial tadpole mash (Xenopus I); according to the authors, food was never completely removed from suspension

Nominal concentrations of the test chemicals were not verified although the authors claim that subsequent studies conducted under similar test conditions allow them to "confidently indicate actual atrazine concentrations likely to have occurred. Atrazine was prepared in acetone (maximum concentration 0.032 ml/L).

Mean mortality in the nitrate and atrazine studies was (0.01 ± 0.01, n=6) and (0.133 ± 0.04, n=4), respectively. There was no significant difference between the different concentrations of the two respective chemicals.

There was no significant difference in time to metamorphosis across nitrate-treated tadpoles (**Table 22**); days to metamorphosis ranged from 48 to 80. However, weight at metamorphosis tended to increase with increasing nitrate concentrations with a significant slope

(p=0.006) associated with the regression analysis slope. Increased weight at metamorphosis was associated with increased time to metamorphosis.

Table 22. Mean days to and weight at metamorphosis. Means with different letters are significantly different based on Tukey's multiple means comparisons.

Treatment	Nominal Concentration	N	Days to Metamophosis	Weight (g)
Atrazine	0 µg/L	25	61.36+5.64 [a b]	0.631+0.132 [c]
	20 µg/L	25	59.96+3.10 [a]	0.517+0.014 [a b]
	40 µg/L	23	60.13+4.30 [a b]	0.527+0.017 [b]
	80 µg/L	30	62.07+4.69 [a b]	0.517+0.019 [a b]
	160 µg/L	24	62.08+4.86 [a b]	0.482+0.017 [a b]
	320 µg/L	29	63.76+4.35 [b]	0.448+0.017 [a]
Nitrate	0 mg/L	30	60.80+1.09 [a]	0.539+0.023 [a]
	10 mg/L	30	60.67+1.15 [a]	0.561+0.027 [a]
	50 mg/L	31	63.03+1.09 [a]	0.546+0.025 [a b]
	100 mg/L	28	62.25+1.15 [a]	0.614+0.028 [a b]
	200 mg/L	31	62.10+0.86 [a]	0.627+0.024 [a b]
	400 mg/L	31	61.29+1.00 [a]	0.664+0.034 [b]

In the atrazine study, tadpoles reached metamorphosis between 53 and 75 days weighing between 0.283 g and 1.03 g. Tadpoles exhibited decreased weight with increasing concentrations of atrazine. Regression analysis indicated a significant slope for atrazine concentration and both days and weight at metamorphosis ($p<0.001$). The authors state that significant regressions were only observed for tadpoles in the 0 and 40 µg atrazine/L treatments for tadpole weight at metamorphosis and time to metamorphosis.

In the mixture study, mortality of tadpoles differed significantly between atrazine treatments (p=0.0018); mortality was significantly greater for tadpoles in the 320 µg/L treatment (0.526±0.075; n=9) compared to controls (0.137±0.032; n=9). Mean mortality did not differ among the nitrate treatments. Based on factorial analysis, there were significant differences in mean values of all tadpole traits (weight, days to metamorphosis, hematocrit, snout-vent length and condition) attributed to atrazine. Tadpoles in distinct nitrate (no atrazine) treatments exhibited no significant effects. Interactions between nitrate and atrazine contributed to significant effects on mean weight and SVL; days to metamorphosis increased with increasing atrazine concentrations (**Table 23**). There were no significant effects of nitrate, atrazine, or the chemicals combined on hematocrit.

Table 23. Mean days to and weight at metamorphosis, hematocrit, snout-vent length (SVL) and body condition (K) for metamorphs exposed to atrazine and nitrate. Means with different footnote letters are significantly different (p=0.05) based on Tukey's multiple means comparisons (all N=3).

Nominal Atrazine (µg/L)	Nitrate (mg/L)	Days to Metamorphosis	Weight (g)	Hematocrit	SVL (mm)	Condition
0	0	74.30±3.68^{bc}	0.920±0.058^{ab}	0.250±0.009	19.34±0.30^{abc}	12.60±0.21^{a}
0	37	65.63±4.25^{c}	1.00±0.012^{a}	0.262±0.010	20.02±0.14^{a}	12.41±0.14^{a}
0	292	70.23±1.51^{bc}	0.924±0.008^{ab}	0.253±0.005	19.53±0.09^{ab}	12.34±0.15^{ab}
40	0	91.09±10.38^{abc}	0.808±0.025^{bc}	0.233±0.014	18.84±0.13^{bcd}	12.09±0.34^{ab}
40	37	84.96±0.86^{abc}	0.765±0.050^{bc}	0.234±0.010	18.55±0.01^{bcd}	11.90±0.15^{ab}
40	292	83.87±5.59^{abc}	0.929±0.036^{ab}	0.251±0.005	19.57±0.17^{ab}	12.34±0.22^{ab}
320	0	89.96±13.49^{abc}	0.718±0.040^{c}	0.251±0.009	18.31±0.23^{cd}	11.06±0.33^{ab}
320	37	100.07±3.04^{ab}	0.669±0.029^{c}	0.224±0.014	18.04±0.14^{d}	11.32±0.32^{b}
320	292	108.15±3.31^{a}	0.722±0.024^{c}	0.220±0.012	18.53±0.21^{cd}	11.27±0.05^{b}

The report concludes that environmentally relevant concentrations of atrazine and nitrate, either singularly or in combination, cause deleterious "ecological" effects on metamorphosis of *X. laevis*. Atrazine exposure resulted in significantly lower body mass, snout-vent length, and body condition at metamorphosis, and these animals required more time to complete metamorphosis relative to controls.

Analysis

The loading rate of 6 tadpoles/500 mL exceeds the ASTM-recommended rate of 1 tadpole/L. Although the methods section says that larvae were transferred to 1 quart Mason jars containing 1.5 L of water, a quart is equivalent to 0.95 L and as such, the Mason jar would be too small to contain 1.5 L of water. The process of combining replicates in this study effectively reduces the ability of the study to discriminate treatment effects. Furthermore, it is unclear how sample size was determined at the end of the study. The treatment unit is the aquarium itself and not the individual metamorphs.

Test animals were fed a commercial diet; however, the study does not report whether any analyses were conducted for pesticides and/or phytoestrogens.

Acetone was used as a co-solvent to prepare atrazine; however, no solvent control was used to determine whether there was a solvent effect. The authors state that previous work has

indicated that acetone at these low concentrations does not affect these aquatic organisms, but data indicating this supposition is not presented. Thus, the study is confounded by the uncertainty of whether, under the conditions tested, the co-solvent may have had an effect.

Although the authors state that atrazine and nitrate concentrations in studies conducted subsequent to the current study could be used to verify concentrations in this study, the claim is difficult to support. Since the exposure chambers were continuously aerated, and the loading rates changed throughout the study, measured concentrations are necessary. Therefore, concentrations discussed in this paper are nominal and have not been substantiated through analytical measurement.

It is unclear from the study whether the reported mortality is indeed less than 1% in all of the studies or whether the reported means are intended as some sort of ratio. Since each replicate in the atrazine alone study was reported to have 6 larvae and each treatment was replicated 5 times, each treatment must have contained 30 larvae. Based on 30 larvae per treatment, then the mortality rate based on the sample sizes for atrazine at 0, 20, 40, 80, 160, and 320 µg/L is 17%, 17%, 23%, 0%, 20%, and 3%. Across all of the treatments, including 0, the average mortality would have been 13%. The authors report the value as 0.133+0.04 (n=6). Sample sizes reported in **Table 22** for the nitrate study exceed the 30 total frogs reportedly used per treatment.

Condition (K) is reported as a measurement endpoint; however, the methods section does not describe how the value is derived.

The mean values reported in **Table 23** are based on a sample size of 3 and is therefore relatively weak (df=2) for distinguishing potential differences between treatments. At face value, the time to metamorphosis appears to be positively correlated with nominal atrazine concentrations in combination with nitrate, *i.e.*, the higher the atrazine concentration, the longer time to metamorphosis; however, for atrazine alone and for nitrate alone the pattern does not appear. For atrazine alone and atrazine in combination with nitrate, there was a tendency toward decreased weight at metamorphosis; however, weight did not appear to be affected by increasing nitrate concentrations alone. Similarly, snout-vent length decreased with increasing concentrations of atrazine and nitrate; atrazine-treatment alone also exhibited a trend for decreasing SVL with increasing atrazine concentration; however, there was no trend in SVL for nitrate alone.

The paper states that the least significant difference test revealed the mean hematocrit value (0.255±0.004, n=9) in treatments receiving 0 µg/L atrazine was significantly greater than the mean values observed in either the 40 or 320 µg/L atrazine treatments, *i.e.*, 0.239±0.006 (n=9) and 0.232±0.008 (n=9), respectively. Since the means are calculated across varying concentrations of nitrate, this may not be an appropriate analysis.

It is not clear from this study whether algae growth in the exposure chambers occurred and if so whether it affected the study. Nitrate may have favored the growth of algae whereas atrazine may have inhibited the growth of algae. For filter-feeding animals like *Xenopus*, differential algae growth may have affected tadpole growth and hence time to metamorphosis.

Animals treated with the highest concentration of atrazine (320 µg/L) on average took 90 days to complete metamorphosis compared to the average of 74 days for controls (**Table 22**). This represents roughly 20% longer. The decreased body weight associated with the highest atrazine treatment was roughly 22% lower than controls. Decreased body weight with increased time to metamorphosis is an unusual finding since longer times to metamorphosis are typically associated with increased body weight as the larvae have longer to feed.

There are confounding effects in this study that limit its utility. It is uncertain whether the increased time to metamorphosis and decreased size at metamorphosis are related to atrazine acting directly on endocrine-mediated processes as the authors suggest or whether the observed effects are due to decreased food availability in the study. Major uncertainties include high loading rates, unverified exposure concentrations in the exposure tanks, and failure to analyze atrazine or phytoestrogens in the feed. The changing exposure conditions and alterations in the number of replicates in this study also seriously compromise the power of this study to differentiate treatment effects. Because of these deficiencies, the study has limited usefulness in determining the potential interactive effects of atrazine and nitrate on metamorphosis of the *X. leavis*

Coady, K. K., M. B. Murphy, D. L. Villeneuve, M. Hecker, P. D. Jones, J. D. Carr, K. R. Solomon, E. E. Smith, G. Van Der Kraak, R. J. Kendall and J. P. Giesy. 2004. Effects of atrazine on metamorphosis, growth, and gonadal development in the green frog (*Rana clamitans*). Journal of Toxicology and Environmental Health, Part A, 67: 941 – 957. (MRID 469656-05).

Description and Results of Study

The purpose of this study was to determine the response of larval green frogs (*Rana clamitans*) to atrazine by assessing metamorphosis and reproductive indices in animals exposed during larval development. The authors conclude that at concentrations ranging between 10 and 25 µg/L, atrazine did not consistently affect growth or metamorphosis. Compared to controls, time to metamorphosis was longer in tadpoles exposed to 10 µg/L of atrazine, but time to metamorphosis was not different from controls among tadpoles exposed to 25 µg/L. Additionally, gonadal morphology in post-metamorphic frogs, based on gross morphology and histology, was not significantly altered in response to atrazine. This study was initially reviewed (USEPA 2003) as an interim report by Hecker *et al*. 2003a (MRID 458677-03). At that time, it was concluded that the high mortality and lack of responsiveness to the positive estradiol controls observed during the study may have been a result of the poor water quality and overcrowding. After a more thorough review of the subsequently published paper (**Appendix 1)**, the underlying concerns regarding this study have not changed from those described in USEPA (2003). The widely fluctuating levels of atrazine in the exposure tanks and the presence of atrazine in the controls seriously confounds the study. The study is further confounded by the very high mortality rates across all exposures groups and the lack of a significant response to one of the two positive controls.

Coady, K. K., M. B. Murphy, D. L. Villeneuve, M. Hecker, P. D. Jones, J. A. Carr, K. R. Solomon, E. E. Smith, G. Van Der Kraak, R. J. Kendall, and J. P. Giesy. 2005. Effects of atrazine on metamorphosis, growth, laryngeal and gonadal development, aromatase activity, and sex steroid concentrations in *Xenopus laevis*. *Ecotoxicology and Environmental Safety* 62: 160 – 173.

Description and Results of Study

The purpose of this study was to further evaluate the effects of chronic atrazine exposure on growth, metamorphosis, and reproductive indices of larval African clawed frogs (*Xenopus laevis*) exposed from 72-hr post-hatch until completion of metamorphosis. Relative to atrazine, the authors conclude that concentrations between 0.1 and 25 µg/L did not significantly affect mortality, growth, gonad development, laryngeal muscle size, or aromatase activity in juvenile *X. laevis*, but that male frogs exposed to 1.0 µg/L had lower estradiol levels compared to controls. This study was initially reviewed (USEPA 2003) as in interim report by Hecker *et al.* 2003b (MRID 458677-04). At that time it was concluded that the protracted development time of all test animals, poor response to the positive estradiol controls, and high variability in the study's measurement endpoints were in large part due to poor environmental conditions and over-crowding. Additionally, fluctuating levels of atrazine in the exposure tanks and the presence of atrazine in the controls further confounded the study. After a more thorough review of the subsequently published paper (**Appendix 1**), the underlying concerns regarding this study have not changed from those previously described (USEPA 2003).

Field Studies

Since the 2003 SAP, a total of eight field studies have been reported in the open literature along with one additional field study submitted by the registrant. All of the field studies represent new versions of studies that were described in interim reports that were reviewed by EPA previously (USEPA 2003). Consequently, only a brief synopsis of each of the studies is provided below. A more thorough review of the study methodologies, results, and the reviewers' analyses is presented in **Appendix 1**.

As discussed in the initial White Paper, field studies provide a means of testing for effects under more natural conditions; however, natural conditions typically increase the extent of variability associated with these studies. Because of the extent to which atrazine has been detected in the environment, most of the field studies have been encumbered by the lack of appropriate reference sites. None of the field studies reviewed prior to the 2003 SAP and none of the more recent studies have adequate reference sites devoid of atrazine, its degradates, or other N-methyl triazine pesticides. Additionally, natural conditions impose additional stressors on test organisms such that it may be difficult to interpret and/or assign cause-effect relationships to the chemical of interest.

As discussed previously (USEPA 2003 and SAP 2003), these reports examined a range of species, *i.e*., *X. laevis*, leopard frogs (*Rana pipiens*), bull frogs (*R. catesbeiana*), and green frogs (*R. clamitans*), in a wide range of regions (Michigan and South Africa). Each of the studies was conducted in the native range for the species; however, sampling at each of the study sites was not equally effective and as a result, sample sizes deviated from the desired N values.

Murphy, M. B., M. Hecker, K K. Coady, A. R. Tompsett, P. D. Jones, L. H. DuPreez, K. R. Solomon, J. A. Carr, E. E. Smith, R. J. Kendall, G. van der Kraak, and J. P. Giesy. 2005. Sediment TCFF-Eq's and EROD and MROD activities in Ranid frogs from agricultural and non-agricultural sites in Michigan (USA). Arch. Env. Cont. Tox. 51(3): 467 – 477. (MRID 469656-01).

Description and Results of Study

The purpose of this study was to measure 7-ethoxyresorufulin *O*-deethylase (EROD) and 7-methoxyresorufin *O*-deethylase (MROD) activities as an indicator of CYP1A6 and CYP1A7 receptor gene expression in Ranid frogs, *i.e.*, adult green frogs (*Rana clamitans*), bullfrogs (*R. catesbiana*) and leopard frogs (*R. pipiens*), collected in agricultural and nonagricultural areas. The study was also designed to investigate site differences with measured concentrations of atrazine in surface waters. In addition, sediment aryl hydroxylase receptor (AhR) activity was determined using a cell bioassay to calculate 2,3,7,8-tetrachlorodibenzo-p-dioxin equivalents (TCDD-EQs) at the study sites.

The authors conclude that of the parameters measured in the study, only liver somatic index values in adult male frogs differed significantly between agricultural and nonagricultural sites, with higher values observed at agricultural sites. Atrazine concentrations were significantly and negatively correlated with MROD activity in adult male green frogs; however, liver somatic index and EROD and MROD activities of adult female and juvenile green frogs were not significantly correlated with atrazine concentrations. Based on this result, the authors state that atrazine does not appear to have a consistent association with EROD or MROD activities in wild-caught green frogs.

This study was initially reviewed (USEPA 2003) as in interim report by Jones *et al.* 2003 (MRID 458677-02). At that time, preliminary data suggested that green frogs were not markedly impacted by atrazine exposure in terms of gonadal deformities. However, some reference sites contained atrazine at levels similar to atrazine sites, and the considerable variability in plasma hormone levels made it difficult to readily detect an effect that could be attributed to atrazine alone. A more thorough review of this paper is provided in **Appendix 1**; the underlying concerns regarding this study have not changed from those described previously (USEPA 2003).

Murphy, M. B., M. Hecker, K K. Coady, A. R. Tompsett, P. D. Jones, L. H. DuPreez, G. J. Everson, K. R. Solomon, J. A. Carr, E. E. Smith, R. J. Kendall, G. van der Kraak, and J. P. Giesy. 2006a. Atrazine concentrations, gonadal gross morphology and histology in ranid frogs collected in Michigan agricultural areas. *Aquatic Toxicology* 76: 230 – 245

Description and Results of Study

The purpose of this study was to determine the incidences of testicular oocytes (TO; single or multiple oocytes found in the testes of male frogs at a histological level) and hermaphroditism (individual frog having both male and female gonad tissue in either one or both gonads at a gross morphology basis) in ranid frogs collected from agricultural and nonagricultural areas and to evaluate correlations between measured atrazine concentrations and these incidences.

The authors state that atrazine concentrations were below the limit of quantification at non-agricultural sites, and concentrations did not exceed 2 µg/L at most agricultural sites; however, at one agricultural site, a measured concentration was greater than 200 µg/L. A low incidence of frogs with both male and female gonad tissue in either one or both gonads (defined as hermaphroditism) were found at both non-agricultural and agricultural sites in both adults and juveniles. Testicular oocytes were found in male frogs at most sites with the greatest incidence occurring in juvenile leopard frogs; however, the incidence was not significantly different between the two types of collection sites with the exception of juveniles collected in 2003. Atrazine concentrations were not significantly correlated with the incidence of hermaphroditism; however, maximum atrazine concentrations were correlated with the incidence of testicular oocytes in juvenile frogs collected in 2003. The authors further state that given the lack of a consistent relationship between atrazine concentrations and the incidence of testicular oocytes, the testicular oocytes observed in the study likely result from natural processes in development rather than from atrazine exposure.

As discussed in the preceding review, this study was initially reviewed (USEPA 2003) as an interim report by Jones *et al.* 2003 (MRID 458677-02). At that time, preliminary data suggested that green frogs were not markedly impacted by atrazine exposure in terms of gonadal deformities. However, some reference sites contained atrazine at concentrations similar to atrazine sites, and the considerable variability in plasma hormone levels made it difficult to readily detect an effect that could be attributed to atrazine alone. A more thorough review of this paper is provided in **Appendix 1**; the underlying concerns regarding this study have not changed from those described previously (USEPA 2003).

Murphy, M. B., M. Hecker, K. K. Coady, A. R. Tompsett, E. B. Higley, P. D. Jones, L. H. Du Preez, K. R. Solomon, J. A. Carr, E. E. Smith, R. J. Kendall, G. Van Der Kraak and J. P. Giesy. 2006b. Plasma steroid hormone concentrations, aromatase activities and GSI in ranid frogs collected from agricultural and non-agricultural sites in Michigan (USA). *Aquatic Toxicology* 77: 153 – 166.

Description and Results of Study

The purpose of this study was to determine whether atrazine exposure is related to plasma estradiol (E2), testosterone (T), and ketotestosterone (KT) concentrations and gonadal aromatase activity in the green frog (*Rana clamitans*), bullfrog (*R catesbeiana*), and Northern leopard frog (*R. pipiens*) collected from agricultural and nonagricultural sites in Michigan.

The authors state that atrazine concentrations did not exceed 2 µg/L at most agricultural sites, but a concentration of 250 µg/L was measured at one site in 2002. Plasma steroid concentrations varied among locations, and aromatase activity was measurable in less than 11% of the adult male testes and in less than 4% of the juvenile male testes. Atrazine concentrations were not significantly correlated with any of the parameters measured in this study, and the authors conclude that atrazine does not up-regulate aromatase in green frogs in the wild and does not appear to affect plasma steroid hormone concentrations.

As discussed in the two preceding reviews (USEPA 2003), this study was initially reviewed as in interim report by Jones *et al.* 2003 (MRID 458677-02). At that time, preliminary data suggested that green frogs were not markedly impacted by atrazine exposure in terms of gonadal deformities. However, some reference sites contained atrazine at levels similar to atrazine sites, and the considerable variability in plasma hormone levels made it difficult to readily detect an effect that could be attributed to atrazine alone. A more thorough review of this paper is provided in **Appendix 1**; the underlying concerns regarding this study have not changed from those described previously (USEPA 2003).

Jooste, A. M. L. H. Du Preez, J. A. Carr, J. P. Giesy, T. S. Gross, R. J. Kendall, E. E. Smith, G. L. Van Der Kraak and K. R. Solomon. 2005. Gonadal Development of Larval Male *Xenopus laevis* Exposed to Atrazine in Outdoor Mesocosms. *Environmental Science and Technology* 39: 5255 – 5261.

Description of Study and Results

The purpose of this study was to determine the effect of atrazine at different concentrations on the number of days to metamorphosis and gonadal development of African clawed frogs, *Xenopus laevis*, up to and beyond Nieuwkoop Faber stage 66 (completion of metamorphosis).

The authors state that, based on histology, 57% of the stage 66 control males exhibited testicular oocytes compared to 57, 50, and 39% of the respective 1, 10 and 25 µg/L atrazine-treated males. The average prevalence of testicular oocytes for all of the treatments including controls was 54% for a single testis and 35% for both testes. The mean number of testicular oocytes was 9.5, 9.8, 8.5, and 11.1 for the 0, 1, 10, and 25 µg atrazine/L groups, respectively. Ten months after metamorphosis, the maximum number of oocytes observed in an individual was five. The authors conclude that the presence of testicular oocytes was not related to atrazine and that testicular oocytes may be a natural phenomenon during individual development (ontogeny).

This study is related to one that was initially reviewed (USEPA 2003) as in interim report by Du Preez and Solomon (2003) (MRID 458677-11). At that time, preliminary data suggested that African clawed frogs reared in the mesocosms exhibited unusually long development periods that may have been related to poor water quality. Additionally, feeding regimes may have limited the growth and/or development of the frogs. A more thorough review of this paper is provided in **Appendix 1**; the underlying concerns regarding this study have not changed from those described previously (USEPA 2003). The paper does suggest that the prevalence of gonadal deformities may best be described through histology rather than gross morphology. The prevalence of gonadal anomalies in males (discontinuous gonads) ranged from 1.7 – 7.1% and 8.8% in controls; however, these results were not correlated with the much larger prevalence of testicular oocytes (up to 57% in the controls) based on histology. The authors report that 7% of the male *X. laevis* collected in the wild were found to have testicular oocytes; however, they also note that few people have conducted in depth histological analysis and may have underestimated the actual prevalence. The high percentage of testicular oocytes reported in the study may be due to environmental conditions in the mesocosms, particularly as they relate to water temperature.

Water temperatures early in the study dropped relatively low for *X. laevis* and may have influenced the extent to which testicular oocytes were present in the adults.

Smith, E. E, L. Du Preez, A. Gentiles, K. R. Solomon, B. Tandler, J. A. Carr, G. Van der Kraak, R J. Kendall, J. P. Giesy and T. Gross. 2005. Assessment of laryngeal muscle and testicular cell types in *Xenopus laevis* (Anura Pipidae) inhabiting maize and non-maize growing areas of South Africa. *African Journal of Herpetology*. 54(1): 69 – 76.

Description and Results of Study

The purpose of this study was to determine whether atrazine and other pesticides are affecting amphibian development by examining morphological changes in the gonads and laryngeal size of African clawed frogs (*Xenopus laevis*) collected in maize-growing regions (MGR) and non-maize-growing regions (NMGR) of South Africa.

The authors state that laryngeal mass was not statistically different between either male or female African clawed frogs from MGR and NMGR sites. Mean percent fractional volume values for seminiferous tubule distribution of testicular cell types (spermatgonia, spermatocytes, spermatozoa, and other cell types) of mature *X. laevis* did not differ significantly between frogs collected from MGR and NMGR sites. The authors conclude that the data demonstrated no significant differences in gonadal and laryngeal development in *X. laevis* collected in South Africa from MGR and NMGR areas and that there is little evidence for an effect of agricultural chemicals used in maize production functioning as endocrine disruptors in *X. laevis*. Additionally, screening of *X. laevis* testes revealed testicular oocytes in 3% and 2% of the adult male frogs collected from NMGR and MGR sites, respectively.

This study is related to two studies that were initially reviewed (USEPA 2003) as interim reports by Smith *et al.* (2003a) (MRID 458677-10) and Smith *et al*. 2003c (MRID 458677-01). At that time, the Agency concluded that any effort to interpret the data from the study would be complicated by the fact that atrazine, atrazine degradates, and other triazine herbicides were detected at significant levels in reference ponds. Although the initial review suggested that a regression-based approach may be more appropriate to examining the potential role of atrazine, the presence of relatively high concentrations of metals in reference ponds may have further compromised the study.

Du Preez, L. H., K. R. Solomon, J. A. Carr, J. P. Giesy, T. S Gross, R. J. Kendall, E. E. Smith, G. Van Der Kraak, And C. Weldon. 2005. Population structure of the African Clawed Frog (*Xenopus laevis*) in Maize-Growing Areas with Atrazine Application Versus Non-maize-growing Areas in South Africa. *African Journal of Herpetology* 54(1): 61 – 68.

The purpose of this study was to determine if there are differences in the sex-ratios, age, and size classes of African clawed frogs, *Xenopus laevis*, inhabiting ponds in a maize-growing area (MGA) versus non-maize-growing area (NMGA) located in the Viljoenskroon and Potchefstroom regions of South Africa.

The authors state that sex ratio, snout-vent length, body weight, and population age structure were similar between MGA and NMGA sites. The authors conclude that there were no significant relationships between exposure to atrazine and any of the parameters investigated in the study.

This study is related to three studies that were initially reviewed (USEPA 2003) as interim reports by Smith *et al.* (2003a) (MRID 458677-10) and Smith *et al*. (2003c) (MRID 458677-01). As stated previously, the Agency concluded that an effort to interpret the data from the study is complicated by the fact that atrazine, atrazine's degradates, and other triazine herbicides were detected at significant levels in reference ponds. Although the initial review suggested that a regression-based approach may be more appropriate to examining the potential role of atrazine, the presence of relatively high concentrations of metals in reference ponds may have further compromised the study.

Hecker, M., J. P. Giesy, P. D. Jones, A. M. Jooste, J. A. Carr, K. R. Solomon, E. E. Smith, G. Van Der Kraak, R. J. Kendall and L. Du Preez. 2004. Plasma Sex Steroid Concentrations and Gonadal Aromatase Activities in African Clawed Frogs (*Xenopus laevis*) from South Africa. *Environmental Toxicology and Chemistry*. 23(8): 1996 – 2007.

Description and Results of Study

The objective of this study was to test the hypothesis that atrazine or related triazines could affect aromatization of testosterone to estradiol under field conditions. In addition, various other measures of water quality (ions, metals, temperature, pH) were examined as potential confounding factors that may interfere with the physiology of the African clawed frog.

In this study, the authors observed that plasma concentrations of both testosterone and estradiol varied among sites and were correlated with some "accessory" factors such as pH, several ions, and metals. The highest median plasma concentrations of testosterone (19 ng/mL in males and 16 ng/mL in females) and estradiol (3 ng/mL in males and 28 ng/mL in females) occurred in frogs collected from non-corn-growing regions (NCGR) as compared to testosterone at 4 ng/mL and 1 ng/mL and estradiol at 2 ng/mL and 5 ng/mL concentrations in males and females, respectively, collected from corn-growing regions (CGR). Since exposure to atrazine occurred at both regions as did simultaneous exposures to multiple chemicals, the authors relied on regression analysis. Based on this analysis, the study identified negative correlations between plasma testosterone concentrations and concentrations of atrazine, deisopropylatrazine, deethylatrazine, and terbuthylazine in females and between plasma testosterone and diaminochlorotriazine in males. Additionally, female plasma estradiol was negatively correlated with atrazine and deethylatrazine. Median ovarian aromatase activity was variable between sites and was up to 3000X greater than median testicular aromatase activity, which was below the level of detection at most study sites. The authors conclude that although exposure to agricultural chemicals did not affect aromatase activities, the potential effects of atrazine or "coapplied pesticides" on plasma steroid homeostasis cannot be discounted.

This study is related to a study that was initially reviewed (USEPA 2003) as interim reports by Smith *et al.* (2003b) (MRID 458677-09). As in the preceding studies conducted in South Africa, the Agency concluded that an effort to interpret the data from the study is complicated by the fact that atrazine, atrazine degradates, and other triazine herbicides were detected at significant levels in reference ponds. Although the initial review suggested that a regression-based approach may be more appropriate for examining the potential role of atrazine, the presence of relatively high concentrations of metals in reference ponds may have further compromised the study.

Smith, E. E., T. A. Anderson, C. Crabtree, J. A. Carr, L. H. Du Preez, A. B. Gentles, J. P. Giesy, T. S. Gross, L. Holden, R. J. Kendall, K. R. Solomon, G. Van Der Kraak. 2007. Reproductive Characteristics in American Bullfrogs (*Rana catesbeiana*) Collected from Agricultural and Non-Agricultural Areas in Southern Iowa. (MRID 472045-01)

Description and Results of Study

The objective of this study was to examine aspects of reproductive status in native North American anuran species, *i.e.,* the bullfrog (*Rana catesbeiana*) collected from agricultural areas (AA) and nonagricultural areas (NAA) in Iowa. Reproductive status was examined through gonadal morphology, plasma testosterone, and plasma estradiol concentrations in relation to atrazine and its metabolite (desethyl atrazine [DEA] and deisopropylatrazine [DIA]) concentrations.

The study indicates that surface water concentrations of atrazine were greater in AA ponds than in NAA ponds and that estimates of gonadosomatic index, sex ratio, and spermatogenesis indicated limited or no differences in these parameters between AA and NAA sites. There were no statistically significant differences in percent fractional volumes of testicular cell types (sperm, spermatocytes, and spermatogonia) of male frogs between AA and NAA sites. Testicular oocytes were only observed in one male collected from AA sites. In this study, there were no statistically significant differences in plasma concentrations of either testosterone or estradiol between males collected from AA and NAA sites, and there was no statistical difference in the sex ratio between frogs collected from AA and NAA sites. In addition, there was no significant relationship between surface water atrazine concentrations and the incidence of gross gonadal deformities or plasma sex steroid concentrations. The authors conclude that since there were biotic and abiotic differences among and between the NAA and AA sites, it wasn't possible to make inferences about the potential causes of the differences in growth, gonadal development,

or plasma sex steroid levels of bullfrogs from NAA and AA sites. The authors considered the differences observed in reproductive characteristics within AA and NAA sites as subtle, and they considered the biological significance of these differences as uncertain.

This published article represents a study by Smith *et al*. (2003) (MRID 458677-05), which was reviewed as an interm report (USEPA 2003). At that time, the Agency concluded that water sampling may not have been sufficient to characterize exposure to atrazine, particularly at reference sites, and no effort was made by the researchers to characterize exposure to other pesticides. The final report provides no additional information to address these uncertainties. Additionally, protracted field collection methods may have impacted the plasma hormone levels that the study was intended to measure. As previously described (USEPA 2003), little information exists on the bullfrog to provide much context for the indices measured in this study. A more thorough review of the study methodologies, results, and the reviewers' analyses is provided in **Appendix 1**.

Gross, T. S. 2007. Characterization of atrazine exposures and potential effects for amphibians inhabiting sugarcane-dominated ecosystems in Florida. Final Report. Ecotoxicology Laboratory, University of Florida at Gainesville. Sponsor: Syngenta Crop Protection, Inc., 410 Swing Rd, P. O. Box 18300, Greensboro, 27419. (MRID 472044-01)

The objective of this study was to characterize the development of the Bidder's organ (rudimentary ovary) and gonads in cane toads (*Bufo marinus*) collected in sugarcane agricultural sites (Cane), non-sugarcane agricultural sites (Agric), and non-agricultural/urban sites (Urban) in southern Florida and to evaluate the potential association between chemical exposure and gonadal abnormalities. This study demonstrates that a higher proportion of males collected from sugarcane agricultural sites had developed Bidder's organ compared to males collected at non-sugarcane agricultural sites and urban sites. All of the males collected exhibited normal testicular tissue. The author notes that increased incidence of developed Bidder's organ in males collected in sugarcane-growing areas should not be interpreted as an association between atrazine exposure since plasma atrazine concentrations were not correlated with the anomaly at any site. It is uncertain whether the development of Bidder's organ in male toads is a natural phenomena or whether it is related to exposure to agrichemical(s) and/or other environmental factors.

This unpublished report represents a study by Sepulveda and Gross (2003) (MRID 458977-06) that was reviewed as an interm report (USEPA 2003). At that time, the Agency concluded that exposure to atrazine and other chemicals was not adequately characterized to

establish a relationship between the incidence of developed Bidder's organs in *B. marinus* and atrazine exposure. The final report provides limited information to address these uncertainties. As previously described (USEPA 2003), little information exists on the cane toad to provide much context for the circumstances that may lead to the development of Bidder's organ in male toads. A more thorough review of the study methodologies, results, and the reviewers' analyses is provided in **Appendix 1**.

ADDITIONAL OPEN LITERATURE

Three additional papers were examined; however, data evaluation records were not completed for the papers since the documents did not represent primary sources of data.

Hayes, T. B. 2005. Welcome to the Revolution: Integrative Biology and Assessing the Impact of Endocrine Disruptors on Environmental and Public Health. Integ. Comp. Biol. 45: 321 – 329.

The purpose of the paper is to review the integration of multiple fields of science that has provided insights into "several" problems in biology such as the role of endocrine disrupting pesticides in amphibian declines. The author cites and reviews open literature studies regarding the potential effects of atrazine on amphibian gonadal development. This paper does not provide any original data. Therefore a data evaluation record was not prepared for this document. Some studies that the author cites were previously reviewed by the Agency (USEPA 2003).

Hayes, T. B. 2004. There is No Denying This: Defusing the Confusion about Atrazine. Bioscience 54(12): 1138 – 1149

The author cites and reviews open literature studies regarding the potential effects of atrazine on amphibian gonadal development. This paper does not provide any original data. Therefore a data evaluation record was not prepared for this document. Some studies that the author cites were previously reviewed by the Agency (USEPA 2003).

Hecker, M., M. B. Murphy, K. K. Coady, D. L. Villeneuve, P. D. Jones, J. A. Carr, K R. Solomon, E. E. Smith, G. Van Der Kraak, T. Gross, L. Du Preez, R. J. Kendall and J. P. Giesy. 2006. Terminology of Gonadal Anomalies in Fish and Amphibians Resulting from Chemical Exposures. Rev. Environ. Contam. Toxicol. 187: 103 – 131.

The purpose of this paper was to provide a brief synopsis of the types of gonadal abnormalities seen in fish and amphibians as a consequence of exposure to endocrine-active compounds in the laboratory and field. This paper does not provide any primary data, but rather constitutes a review of previously published observations.

CHAPTER 4

Since the 2003 SAP, the Agency has identified and reviewed 19 studies that address the effects of atrazine alone on amphibian gonadal development. **Table 24** summarizes the studies reviewed in this White Paper and provides a brief list of the major findings and limitations associated with each of the studies. Below is a brief discussion of the strengths and limitations of each of the studies.

STUDY STRENGTHS AND LIMITATIONS

LABORATORY STUDIES IN RESPONSE TO DCI

The basis for the two studies conducted in response to the DCI was a study protocol recommended by both EPA (USEPA 2003) and the 2003 SAP (SAP 2003). The protocol was intended to address various sources of variability that were evident in the studies reviewed for the 2003 SAP. These studies endeavored to maintain optimal environmental conditions through the use of flow-through systems, reduced tadpole loading rates, and more appropriate feeding regimes. In spite of these efforts, some of the tanks at one of the test facilities failed because of algal growth, and these replicates had to be dropped from the study. Additionally, one cluster of negative control tanks was determined to be contaminated with atrazine at the level of quantification (0.01 µg/L); contamination within this cluster continued for several weeks and these tanks were dropped from the analysis. Positive estradiol controls in one study were inadvertently contaminated with atrazine (0.1 µg/L) during week 6 of the study.

The researchers recorded a number of measurement endpoints and several of these (unpigmented ovaries, translucent gonadal tissue, diameter of the ovarian cavity) demonstrated varying degrees of statistical significance relative to the controls. However, the relationship of these secondary measurement endpoints to apical endpoints (time to metamorphosis, size at metamorphosis, sex ratio, and the incidence of intersex/mixed sex gonads) may or may not have biological significance. This question cannot be answered at this time. .

Table 24. Summary of laboratory and field study findings and study deficiencies.

Study	Test Species	Developmental Stage	Atrazine Concentration (ppb)	Major Results	Study Deficiencies
Laboratory Studies					
Hosmer *et al*. 2007	*Xenopus laevis*	NF Stage 46 - 66	0.01 - 100	Size at metamorphosis not affected consistently; body weight and length significantly lower at 0.01, 1, and 100 ppb. No testicular oocytes/mixed sex observed in atrazine-treated animals. Time to metamorphosis not affected.	Atrazine contamination of controls; measured atrazine concentrations below nominal; marked fluctuations in water quality parameters; histological endpoint comparisons not made relative to amphibian reference.
Freeman and Rayburn 2005	*Xenopus laevis*	NF Stage 47 - 58	100 - 800	Delayed developmental stage; however, time and size at metamorphosis not measured.	Lack of a consistent dose response within and/or between labs. Time to and size at metamorphosis not reported. No information provided on development of gonads.
Hayes *et al*. 2006a	*Xenopus laevis*	NF Stage 50 - 66	0.1 – 25	Single sex polygonadalism (males); unpigmented ovaries; hermaphroditism (males).	Animal husbandry conditions (plastic tanks, high loading rates) may have affected development; study results inconsistent with described methods. Sex ratio of controls skewed. Lack of a consistent and clear dose response. Water quality parameters not reported.
Hayes *et al.* 2006b	*Rana pipiens* ---------- *Xenopus laevis*	Gosner Stage 21 – 46 ---------- Adult	0.1 – 10 ---------- 0.1	No gonadal abnormalities; no effect on time to and size at metamorphosis. ---------- Thymic plaques; immunosuppression	Water quality parameters not reported; assay limit of detection and quantification not reported. Animal husbandry conditions (plastic mouse cages) may have affected development. Measured concentrations not reported for all treatments; lowest nominal concentration is at limit of detection. Reported results are inconsistent with the data presented in report. Authors rely on the lack of statistical significance to draw conclusions.
Hecker *et al*. 2005a	*Xenopus laevis*	Sexually mature adults	1 - 250	No gonadal abnormalities. Decreased plasma testosterone at 250 ppb atrazine; no consistent dose respone.	Construction of exposure chambers not reported. Relatively high concentrations of atrazine tested. Sexually mature adult frogs used; therefore, study does not provide information on the effects of atrazine on time and/or size at metamorphosis nor does it provide information on the effects of atrazine on gonadal development.

Study	Test Species	Developmental Stage	Atrazine Concentration (ppb)	Major Results	Study Deficiencies
Hecker *et al*. 2005b	*Xenopus laevis*	Sexually mature adults	10 - 100	No gonadal abnormalties; no affect on plasma estradiol or testosterone concentrations. GSI significantly higher in animals treated with 10 ppb atrazine, but no consistent dose response.	High loading rates; fiberglass exposure chambers; water quality parameters not reported. No solvent control run for positive contol. Sexually mature adult frogs used; therefore, study does not provide information on the effects of atrazine on time and/or size at metamorphosis nor does it provide information on the effects of atrazine on gonadal development.
Orton *et al*. 2006.	*Rana pipiens*	Taylor-Kollros Stage 2/3 – 23.24	10	Testicular oocytes in atrazine-treated frogs; however, no dose-response and incidence of intersex in controls was higher than in any of the treatments. Sex ratio (male:female) higher in atrazine treated; however, control sex ratio was unusually low. Decreased percentage of spermatocytes and increased percentage of spermatids in testes; increased ovarian follicle size in females.	High loading rates; high control mortality; high incidence of testicular oocytes in controls; sex ratio of controls appears skewed.
Sullivan and Spence 2003.	*Xenopus laevis*	NF Stage 46 - 66	20 – 320	Decreased weight and length at metamorphosis and increased time to metamorphosis.	High loading rates; no solvent control; nominal concentrations not verified through measurements. Changing exposure conditions and alterations in the number of replicates.
Coady *et al.* 2004	*Rana clamitans*			Increased time to metamorphosis at 10 ppb; no consistent dose response.	High loading rates; high mortality; lack of responsiveness to positive control (estradiol).
Coady *et al.* 2005	*Xenopus laevis*		0.1 - 25	No effect on mortality, growth, gonad development, laryngeal muscle size or aromatase activity; males at 1.0 ppb have lower serum estradiol concentrations; no consistent dose response.	High loading rates; high mortality; lack of responsiveness to positive control (estradiol).
Field Studies					
Murphy *et al*. 2005	*Rana clamitans* *Rana catesbeiana* *Rana pipiens*	Adult	variable	Liver somatic index higher in agricultural sites; atrazine concentrations negatively correlated with MROD activity in male *R. clamitans*.	Atrazine identified at all of the study sites including reference sites; high variability in plasma hormone levels.
Murphy *et al*. 2006a	*Rana clamitans*	Juvenile – Adult	variable	Atrazine not significantly correlated with incidence of hermaphroditism; however, atrazine concentration correlated with the incidence of testicular oocytes in juvenile green frogs collected in one of the two years.	Atrazine identified at all of the study sites including reference sites; high variability in plasma hormone levels.

Study	Test Species	Developmental Stage	Atrazine Concentration (ppb)	Major Results	Study Deficiencies
Murphy *et al.* 2006b	*Rana clamitans Rana catesbeiana Rana pipiens*	Juvenile - Adult	variable	Atrazine exposure not correlated with aromatase activity nor with plasma steroid concentrations in green frogs.	Atrazine identified at all of the study sites including reference sites; high variability in plasma hormone levels.
Jooste *et al.* 2005.	*Xenopus laevis*	NF Stage - 10 months post-metamorphosis	0.1 - 25	Atrazine exposure not correlated with testicular oocytes; testicular oocytes may be a natural phenomenon.	Unusually long development times; feeding and temperatures may have limited development. High incidence of testicular oocytes in controls
Smith *et al.* 2005.	*Xenopus laevis*	Adults	variable	No significant differences in gonadal or laryngeal development relative to atrazine exposure; incidence of testicular oocytes ≤3%.	Relatively high levels of atrazine and atrazine degradates in reference sites; relatively high concentrations of metals at reference sites.
Du Preez *et al.* 2005	*Xenopus laevis*	Adults	variable	No significant differences in sex ratio, size (length/weight) or population age structure relative to atrazine exposure.	Relatively high levels of atrazine and atrazine degradates in reference sites; relatively high concentrations of metals at reference sites.
Hecker *et al.* 2004	*Xenopus laevis*	Adults	variable	Negative correlation between plasma testosterone and atrazine, its degradates and terbuthylazine in females; female plasma estradiol negatively correlated with atrazine and desethylatrazine. Negative correlation between male plasma testosterone and diaminochlorotriazine.	Relatively high levels of atrazine and atrazine degradates in reference sites; relatively high concentrations of metals at reference sites.
Smith *et al.* 2007	*Rana catesbeiana*	Adults	variable	One animal found with testicular oocytes; no significant correlations between atrazine exposure and the incidence of gross gonadal deformities or plasma sex steroid concentrations.	Insufficient characterization of atrazine at study sites; insufficient characterization of other chemicals at study sites. Protracted field collection may have impacted plasma steroid levels.
Gross *et al.* 2007	*Bufo marinus*	Adults	variable	Increased incidence of developed Bidder's organ in males collected in sugarcane-growing regions where measured atrazine concentrations were highest.	Atrazine and other chemicals not sufficiently characterized at all study sites. Collection methods not sufficiently described; age of test animals not sufficiently described. Some of the measurement endpoints were below the method level of detection.

In 2003, the Agency recommended that Tier I studies focus on demonstrating whether atrazine exposure results in effects on amphibian gonadal development in *X. laevis*. The studies in response to the DCI have relied on the African clawed frog as a test species. Although the 2003 SAP could not provide information to suggest that *X. laevis* should not be considered a reasonable surrogate for other amphibians, there is uncertainty whether an amphibian species indigenous to North America may have responded differently.

Contrary to what the Agency recommended in 2003, the current studies did not measure the concentration of the major atrazine degradates, *i.e*., DACT, DEA, and DIA. Measured atrazine concentrations deviated from nominal throughout the course of the study and tended to be less than nominal. As the study progressed and animals were removed from the treatment units at completion of metamorphosis, measured concentrations better approximated nominal concentrations. Since measured concentrations in stock solutions were >90% of nominal and since metering devices were functioning properly, the decreased concentrations of atrazine in the treatment units was likely due to uptake by the test animals. As mentioned previously, the measured concentrations did span five orders of magnitude and reflected a broad range of atrazine exposure concentrations. While measured concentrations deviated from nominal, actual exposure was documented throughout the study.

Also contrary to previous recommendations, the studies did not enumerate specific cell types during the histological analysis. Differential cell counts were not conducted because there were an insufficient number of morphologically distinct cell types since the gonads of the juvenile (NF Stage 66) where the majority of cells within the ovaries and testes were gonadal germ cells (pers. comm.: Dr. Jeff Wolfe, Pathologist, EPL, 2007). The number of specific cell types may be more closely related to chronological age (days post-hatch) as opposed to NF-development stage and the study animals were not of uniform chronological age since time to metamorphosis varied across test animals. Additionally, pilot studies with estradiol indicated that other histological features, *e.g.* dilated tubules and dividing gonadocytes in the testes and changes in ovarian cavity size in ovaries, were more sensitive measures of estrogenic effects. Finally, given the number of tissue sections evaluated, the time required to conduct differential cell counts would have greatly extended the projected study completion date.

Several of the secondary gonadal observations made at the histological level referred to the control condition. EPL's glossary of histopathological diagnoses specifically defines

numerous observations as relative to the controls, including: increased apoptotic bodies (testes), decreased germ cells (testes and ovaries), hypoplasia (testes and ovaries), increased oocyte atresia, decreased melanophores (ovaries), interstitial fibrosis (ovaries), decreased ovarian cavity, and increased ovarian cavity. However, since the study was read in a manner that the treatment was unknown to the pathologist, it is uncertain how the relative comparisons were made. Therefore, the validity of these observations is questionable.

Although the studies in response to the DCI had shortcomings with respect to atrazine and estradiol exposures (*i.e.*, measured concentrations were less than nominal and a cluster of control tank replicates had to be discarded because of atrazine contamination), the study appeared to be sensitive to detecting the effects of chemicals on amphibian gonadal development as evidenced by the responsiveness of positive control animals. Overall, environmental factors were controlled and optimized sufficiently to reduce factors that had confounded previous studies to determine whether atrazine exposure can affect amphibian gonadal development. While there were shortcomings in the execution of aspects of the study, the Agency concludes that they are not sufficient to preclude a sufficiently robust assessment of the hypothesis. The basis for this conclusion is that exposure to atrazine was well documented throughout the study; control animal growth, development, and survival was consistent with established standards based on Nieuwkoop and Faber (1994) and demonstrated that environmental conditions were optimized. Additionally, responses to positive controls were consistent with what has been reported in open literature (Hayes 1998) and demonstrated that the experimental design could detect effects on amphibian gonadal development.

OPEN LITERATURE LABORATORY STUDIES

Since many of the studies reported in the open literature were the finalized versions of interim reports reviewed for the 2003 SAP, the same limitations identified in the 2003 White Paper apply. These limitations include:

- poor control of environmental conditions and study animal husbandry factors capable of influencing endpoints the studies were attempting to measure;
- absence of standardized protocols that would allow other researchers to replicate the methods; and
- inadequate study designs to overcome high variability leading to poor statistical power to differentiate treatment effects.

Exposure chambers were constructed of a variety of materials including plastic (Hayes *et al*. 2006a; Hayes *et al.* 2006b) and fiberglass (Hecker *et al*. 2005b); the potential effect of these construction materials on the study is uncertain. High loading rates coupled with incomplete (50%) and/or infrequent (every 72 hours) exposure solution changes continued to be a major limitation in the majority of the laboratory studies reported in the open literature (Hayes *et al* 2006a; Hayes *et al.* 2006b; Hecker et al. 2005b; Orton *et al.* 2006; Sullivan and Spence 2003; Coady *et al*. 2004; Coady *et al.* 2005).

None of the laboratory studies reported any analyses conducted on the commercial diets fed to test animals. Whether these commercial diets contained other pesticides such as atrazine or phytoestrogens, which may have impacted the study and contributed to variability, is uncertain.

Additionally, exposure conditions were frequently characterized poorly, and atrazine was detected in controls (Coady *et al*. 2004; Coady *et al*. 2005) of many of the studies at concentrations at or approaching actual treatment levels. Thus, although laboratory studies are intended to provide an opportunity to control potential sources of variability that could affect the endpoints of interest, none of the experiments reported in the open literature fully accounted for environmental conditions and husbandry factors capable of influencing endpoints the studies were attempting to measure. Once again these problems could have resulted in unfavorable environmental conditions for optimum growth and development, and in fact, reported growth and development of unexposed organisms were inconsistent with patterns cited in the open literature (Nieuwkoop and Faber 1994).

Generally, the plasma hormone concentrations and gonad/brain aromatase activity assays were characterized by extremely high levels of variability (Hayes *et al*. 2006b; Orton *et al*. 2006; Coady *et al*. 2005). Coefficients of variation ranged as high as 550%, indicating that the means to detect a treatment effect was problematic. In some cases, hormone assays could not distinguish males from females. The variability in these measurement endpoints may have been related to handling stress and protracted sampling periods.

Although most of the studies employed positive controls using dihydroxytestosterone and estradiol, typically there was a lack of responsiveness to these hormones (Coady *et al*., 2005; Coady *et al*. 2004), implying that hormone concentrations were not sufficiently high, or that the

test animals were not responsive to the chemicals, or that there were technical problems with the analytical methods used to measure sex steroid. According to an open literature study, *X. laevis* treated with estradiol 72-hrs post-hatch through metamorphosis should produce 100% females (Hayes 1998); however, in the current battery of studies, estradiol treatment only yielded a maximum of 67% females and in some studies did not have any effect on sex ratios. Furthermore, measured estradiol concentrations in some of the studies were considerably lower than nominal. While it is possible that some species were not responsive to hormone treatments, it is uncertain why these species would be selected as appropriate measures of estrogenic/androgenic effects. The current open literature studies suggest that poor water quality, overcrowding, and insufficient hormone concentrations may have contributed to the lack of responsiveness for many of the animals to estradiol and dihydroxytestosterone.

The poor condition of the study animals was a recurrent theme in the majority of the open literature laboratory study reviews. With mortality rates as high as 75% across all treatments in one study and disease in another, the viability of the test organisms was clearly in question (Orton *et al*. 2006; Sullivan and Spence 2003; Coady *et al*. 2004). The protracted length of time required for the frogs to undergo metamorphosis (80+ days as opposed to the more typical 58-day period for *X. laevis*) and the tendency for animals to lose weight with increasing length of time to metamorphosis underscored EPA's concerns that study conditions were not conducive to optimal growth. The decreased condition and growth could have impacted the developmental status of the animals as evidenced by the number of frogs that did not complete metamorphosis.

In several of the studies, conclusions were drawn on what appears to be a misinterpretation of the significance of the statistical tests used. Additionally, in at least one study (Hayes *et al*. 2006b), the authors draw conclusions from the lack of statistical significance.

In the most recent study by Hayes *et al.* 2006, the authors fail to replicate their previous work and show instead that atrazine exposure does not result in gonadal deformities in frogs. The authors attribute this unresponsiveness to biological variability of Northern leopard frogs across the studies. Effects reported in this study are no longer attributed to the effect of atrazine on gonadal amphibian development but rather to the effects of pesticide mixtures on immunosuppression.

OPEN LITERATURE FIELD STUDIES

As mentioned previously, field studies can help evaluate the relevancy and significance of toxicological effects observed in laboratory-based investigations. Unlike laboratory studies, however, the more natural conditions of field experiments can add variability to the data. Consequently, considerable effort is required to assure that natural variability across field study sites is accounted for and that sites do not have characteristics capable of interfering with the interpretation of the data. Most of the studies reviewed, however, did not provide sufficient information to characterize study sites or provide sufficient rationale for site selection and variability. Atrazine exposure in reference/control sites, with likely exposure to a much wider array of chemicals, reduced if not eliminated the option of conducting hypothesis testing and led some investigators to consider a regression-based approach, even though the study design was not originally intended to support such an analysis. In addition, the evaluation of potential confounding effects of non-chemical stressors, *e.g*., habitat condition, prey availability, nutrient loading, were not described or evaluated in these studies.

While the difficulty in selecting field sites that have similar morphological characteristics is well recognized and appreciated, many of the study sites had widely divergent conditions. Although atrazine is frequently detected in monitoring studies, the potential for exposure of field study reference sites was not adequately considered in the majority of studies. Quantifiable levels of atrazine and/or its degradates were detected in many of the studies (Murphy *et al*. 2005, Murphy *et al*. 2006a; Murphy *et al*. 2006b; Smith *et al*. 2005; Du Preez *et a*l. 2005; Hecker *et al*. 2004). Although some of the study authors conducted pilot studies to establish atrazine levels in experimental and reference sites, follow-up monitoring suggests that the initial surveys were misleading, *i.e*., atrazine concentrations in reference sites were considerably higher than initial sampling indicated. Additionally, other triazine herbicides (*e.g*., simazine, terbuthylazine) were detected in reference sites at comparable if not higher levels than at atrazine-exposed sites. Although other pesticides were admittedly used in the vicinity of many study locations, none of the pesticide concentrations were characterized.

Ideally, field studies should be designed based on the variability associated with the measurement endpoints, and 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 studies did not appear

to be designed based on the variability associated within the range of measurement endpoints. In some cases, animals were collected over extended periods of time (up to 6 months), which could increase variability due to differences in developmental stage and reproductive status of the organisms at the time of collection. Since none of the field studies adequately documented prior exposure history of test animals nor did they adequately account for the potential confounding effects of environmental conditions and possible exposure to other chemicals/metals, the field studies provide limited basis for testing the hypothesis that atrazine affects amphibian gonadal development.

In some cases sampling extended over several hours to many months and may have resulted in a comparison of animals that were at different stages of their sexual maturation cycles (Smith *et al*. 2007). Additionally, *X. laevis* is an opportunistic breeder, and it is unclear what environmental factors initiate breeding responses. Placing males and females in close proximity to one another during collection could potentially impact plasma sex hormone levels.

As noted in the 2003 SAP report (SAP 2003), field studies failed to measure responses for which field studies are most revealing. Whereas abundance and age structure were measured occasionally, endpoints related to reproduction, recruitment and populaton viability were absent. Additionally, most of the field studies were observational and no effort was made to contol potentially confounding factors. The preponderance of field studies evaluated since the 2003 SAP indicate that atrazine was not consistently correlated with the incidence of testicular oocytes in amphibians. The study by Gross (2007) was an exception though; however, the exposure history of *B. marinus* to atrazine and other chemicals is poorly characterized in the study by Gross (2007) and the natural incidence of developed Bidder's organ in males is not well documented.

CHAPTER 5

CONCLUSIONS

In June 2003, the Agency evaluated the available literature on the potential effects of atrazine on amphibian gonadal development and concluded that while there was sufficient evidence to formulate a hypothesis that atrazine exposure can affect amphibian gonadal development, there was insufficient evidence at that time to confirm or refute the hypothesis, in large part due to the limitations of the study designs and uncertainties in the data. Consequently, the Agency made recommendations (USEPA 2003) regarding study designs necessary to address the hypothesis. The Agency also proposed (USEPA 2003) a tiered-study approach. The FIFRA SAP (SAP 2003) concurred with the Agency's interpretation of the available data and with the Agency's recommendation for additional data. Subsequent to the 2003 SAP, the Agency issued a data call-in (DCI) as part of its interim reregistration eligibility decision on atrazine and required the technical registrant to complete the first phase of testing to establish whether there is a causal relationship between atrazine exposure and amphibian gonadal development in African clawed frogs (*X. laevis*).

In response to the DCI and in consultation with the Agency, the registrant developed a study protocol. Since 2003, new data have been generated. The registrant completed two independent experiments following the same study protocol, and the results of those experiments with the supporting data, were provided to the Agency in 2007. In addition to the studies conducted in response to the DCI, the Agency identified and reviewed studies reported in the open literature since the 2003 SAP report (SAP 2003) and has reviewed those studies. The data from the Syngenta studies and the open literature form the basis for EPA's conclusions.

Based on recommendations described in the 2003 White Paper (USEPA 2003) as well as from the FIFRA SAP (SAP 2003), the registrant undertook two experiments to determine whether atrazine exposure affects apical measures of gonadal development in *X. laevis*. Exposures were conducted using free-swimming larvae at developmental stages (NF Stages 46 – 54) known to be sensitive to feminization by exogenous estrogens through metamorphosis (NF Stage 66). Environmental conditions favoring growth, survival, and development were maximized through the use of flow-through systems and adequate feeding regimes. Loading rates (1 tadpole/L/day)

were consistent with ASTM-recommended levels to prevent overcrowding and to maintain optimum environmental conditions.

Also consistent with the recommendations made by EPA and the FIFRA SAP, 17β-estradiol "positive" controls were used as a means of demonstrating the sensitivity of the test organism to a potential estrogen effect.

Sampling of the test organisms for apical effects occurred at the same developmental stage, *i.e.*, NF Stage 66, and was consistent across the entire study. Endpoints measured were consistent with recommendations that included gross and histological gonadal morphology to determine whether effects such as testicular oocytes and reduced spermatogonial cell nests occur in males and whether alterations in the numbers of primary and secondary oogonia occurred in females. Additionally, male:female sex ratios, observations on secondary sexual characteristics and on daily survival, growth (as determined by wet weight and snout-vent length at metamorphosis), and development (as indicated by time to complete metamorphosis) were evaluated.

Based on the available data, the study appears to have met the quality indicators stipulated in the 2003 SAP. Abiotic factors were maintained at reasonable levels. Dissolved oxygen was maintained between 60 – 100% of saturation, pH was between 7.5 to 8.5, and total ammonia was well below 10 ppm. The study maintained a loading rate consistent with the ASTM-recommended 1 g/L/day; survival was greater than 90% across all treatments and controls; body weights at metamorphosis fell within the historic range (0.42 – 0.83 g) for NF Stage 66 frogs; and test animals completed metamorphosis within the 7 – 9 week timeframe identified in the 2003 White Paper (USEPA 2003).

Given that the study adhered to the recommended design elements and met the quality criteria established by the both EPA (USEPA 2003) and the FIFRA SAP (SAP 2003), the study is viewed as a reliable means of detecting effects on the intended apical endpoints.

Both of the studies conducted by the registrant had limitations that included inconsistent exposure concentrations that fluctuated from nominal concentrations and atrazine contamination of some control tanks. Although these factors could have raised questions about the validity of a study, the rigor and quality control under which the recent studies were conducted provided

documentation on actual exposure throughout the study. Additionally, the documentation provided evidence that the contamination of control tanks with atrazine was limited to a cluster of tanks juxtaposed between tanks at the lowest and highest (100 µg a.i./L) treatment concentration. Since twice the number of control tanks (16 tanks) was used than treatment tanks (8 tanks), the cluster of tanks containing atrazine residues could be discarded without affecting the quality control or analysis of the study results.

Based on the studies conducted at IGB and WLI, atrazine exposure concentrations ranging between 0.01 and 100 µg/L did not result in any consistent statistically significant effect on mortality, time to metamorphosis, size at metamorphosis or on any of the indices used to gauge amphibian gonadal development. Abnormalities in amphibian gonads such as testicular oocytes and intersex were limited to positive estradiol control animals alone where the percentage of mixed sex animals ranged from 6 to 18% across the two studies. In addition to the occurrence of mixed sex animals, positive controls also exhibited a statistically significant increase in the number of females; females comprised 81 and 78% of the animals in the WLI and IGB studies, respectively. This skewed sex ratio of the positive control is consistent with the intent of the study to use the estradiol median effect concentration (EC_{50}) for sex ratio.

A number of measurements based on gross morphology were reported as being statistically significant (gonadal image area, gonadal segmental translucence, gonadal hypoplasia and segmental hypoplasia, and segmental hypertrophy); however, the frequency of the effects was relatively low and the effects were typically observed only during gross morphological exam and not during histology. Statistical significance was often detected in only one of the two study labs, and the biological relevancy of the measurement endpoints is not clear since apical endpoints did not appear to be affected. Additionally, the terminology used to describe gross morphological features such as hypertrophy (increased size), hypoplasia, and aplasia (decreased size) were intended to describe the general size of the organ rather than to attribute a specific cause such as increased number/size of cells or decreased number/size of cells. The underlying explanation for decreased size of the organ could only be determined from histology and not gross morphology. Therefore, the biological significance of gross morphological observations is uncertain.

An even larger number of histopathological evaluation endpoints were developed, and several of these were statistically significant compared to control animals, although variability in the data was considerable. Trend test analyses also indicated significance for several of the

endpoints. Only one of the histopathological evaluation measures (fused kidneys) exhibited a significant trend across both laboratories at the highest atrazine concentration tested, *i.e.*, 100 µg/L. As stated previously, the biological relevancy of the gross morphological data reported in the studies is not apparent unless they can be linked to some histopathology, which in turn can be linked to more apical endpoints.

The open literature studies continue to exhibit many of the same sources of variability that limited the utility of data as discussed in 2003 (USEPA 2003; SAP 2003). Conflicting and contradictory results confounded by poorly controlled experimental conditions render these data of little value for testing the hypothesis. While the studies suggest possible areas for additional research, they do not account for the previously identified sources of variability and preclude the means to test the hypothesis.

The lack of a reproducible dose-response relationship and the lack of reproducible effects in these studies are viewed as major limitations. The DCI studies conducted in response to the recommendations made by EPA (USEPA 2003) and concurred to by the FIFRA SAP (SAP, 2003) indicate that across a wide range of atrazine concentrations that extend well beyond the gamut of measured environmental concentrations as discussed in the IRED (USEPA 2003c), atrazine does not affect amphibian gonadal development in African clawed frogs. Additionally, the data indicate that atrazine exposure does not affect time to metamorphosis, and size at metamorphosis nor does atrazine exposure affect sex ratios. The studies conducted in response to the DCI were designed to address uncertainties and potential sources of variability that limited the utility of previous studies. Because of the experimental design protocols and quality control of the DCI studies, the data are sufficiently robust to outweigh previous efforts to study the potential effects of atrazine on amphibian gonadal development.

Based on the negative results of these studies, the Agency concludes that it is reasonable to reject the hypothesis formulated in the 2003 SAP that atrazine exposure can affect amphibian gonadal development. The Agency believes at this time, there is no compelling reason to pursue additional testing with regard to the potential effects of atrazine on amphibian gonadal development.

With respect to the uncertainty that other amphibian species may respond differently to atrazine than those that were tested in the DCI studies, the Agency does not believe that further

testing is warranted. In 2003, the SAP (SAP 2003) indicated that *X. laevis* is a reasonable test species for evaluating the potential effects of atrazine on amphibian gonadal development. Additionally, the SAP (SAP 2003) indicated that there was little to no evidence to demonstrate significant differences in developmental pathways between indigenous species such as *Rana spp,* and *Xenopus* that would preclude the use of *Xenopus* as a model in future studies.

CHAPTER 6

CHARGE TO THE PANEL

The Agency has reviewed relevant studies in the scientific literature published since 2003, as well as studies submitted by the registrant in response to a DCI, to evaluate the potential for atrazine alone to elicit effects on amphibian gonadal development and differentiation. The strengths and limitations of the individual studies were assessed, and the extent of concordance for the entire body of information derived from these laboratory and field studies was considered to assess the plausibility that atrazine can cause developmental effects in amphibian gonads, and if so, the nature and strength of associated dose-response relationships. This analysis, while primarily focused on gonadal development and differentiation, included data on other developmental effects, including: time to metamorphosis, growth, sex ratios, and gonadal abnormalities.

The Agency is seeking comments and recommendations from the SAP on a number of questions surrounding the current body of evidence regarding the potential effects of atrazine on gonadal development in amphibians and the relevancy of these potential effects to an ecological risk assessment of atrazine. The Agency is also seeking input from the SAP on its conclusion that, based on the results of the most recent studies in response to the DCI, atrazine exposure does not affect amphibian gonadal development and that no further testing is required to address this hypothesis.

Questions

1) In reviewing the available laboratory and field studies, the Agency used a number of criteria to evaluate individual investigations. Criteria such as experimental design, test protocols, and quality assurance information were used to evaluate the reliability of a study's ability to adequately assess a hypothesis that atrazine elicits developmental effects in amphibians, and if so, the nature and strength of associated dose-response relationships.

(a) Please provide comments and recommendations regarding the EPA's approach and criteria used to evaluate the studies.

(b) Given the evaluation criteria employed by the Agency, please comment on EPA's overall application of these criteria to the currently available studies.

Questions Concerning the Open Literature Studies

2) The Agency has concluded that the information contained in the open literature published since the 2003 SAP does not provide any additional information that could be used to refute or confirm the hypothesis that exposure to atrazine alone causes adverse developmental effects in amphibian gonads.

(a) Please comment on the comprehensiveness of the Agency's literature reviews relative to the potential effects of atrazine alone on amphibian gonadal development.

(b) Please comment on the Agency's evaluation of the open literature studies and the Agency's conclusion that the data derived from laboratory studies, both individually and collectively, are not sufficient to refute or confirm the hypothesis that atrazine exposure causes developmental effects in amphibian gonads.

(c) The Agency concluded that the field studies are not adequate for assessing the hypothesis at hand. Please comment on the Agency's conclusion. If the SAP concludes one or more of the field studies do provide the means to assess the hypothesis that atrazine exposure results in effects on amphibian gonadal development, please suggest interpretive and statistical methods that should be employed to evaluate the data.

Questions Concerning the DCI Study

3) Please comment on the Agency's evaluation of the final study design. For example, the Agency concluded that the minor changes in the experimental design [*i.e.*, omitting atrazine degradate (DACT, DEA and DIA) analysis and not conducting differential cell counts for ovarian and testicular histology] did not compromise the means to assess the hypothesis that atrazine exposure can affect amphibian gonadal development. If the SAP concludes that the alterations in the study design preclude or significantly compromise the ability to assess the hypothesis, please discuss to the extent possible, how the specific design modifications could impact the means to assess the hypothesis. Please provide comments on other aspects of the Agency's evaluation as well.

4) The Agency has described the exposure profiles for studies conducted in response to the DCI and has stated that mean-measured concentrations in the studies were lower than target nominal concentrations. However, the Agency concluded that the frequent

analytical measurements provide a sufficiently comprehensive understanding of the exposure profile over the course of the studies. Please comment on the Agency's conclusion that the atrazine exposure concentration profile is reasonably characterized and sufficient for documenting the potential effects of atrazine over a broad range of exposure concentrations. In addition, provide comments on whether the actual concentrations were consistent and sufficiently stable to establish the means to analyze exposure concentration-response relationships.

5) The Agency described atrazine contamination of negative controls in one out of the two studies and concluded that since the experimental design had twice the number of controls relative to other treatments, the data from these atrazine-contaminated controls could be removed from the analyses without invalidating the statistical interpretation of the results.

(a) Please comment on the Agency's decision to omit half of the controls from the WLI study in the statistical analyses and on the conclusion that the study is still scientific valid. If the SAP has an alternative approach to treating these control data in the statistical analyses, please provide specific recommendations.

6) The original White Paper (USEPA 2003) identified measurement endpoints that included the possible enumeration of specific histological structures such as the number of oogonia in ovaries and the number of spermatids in testes. Such a detailed analysis was not conducted in the studies that are in response to the DCI. Rather, a qualitative assessment of the incidence of ovarian and testicular gonadocytes was conducted. The Agency has concluded that the lack of these data does not limit the means to assess the hypothesis that atrazine exposure affects amphibian gonadal development.

(a) Please comment on the whether the lack of these histological data limits the utility of the available information to fail to support the hypothesis that atrazine exposure affects amphibian gonadal development.

(**b**) If the SAP concludes these data are necessary to adequately assess the hypothesis, please provide options to processing and analyzing these data in an efficient and robust manner.

7) The Agency has described a number of measurement endpoints (*e.g*., translucent gonads, unpigmented ovaries, pigmented testes) based on histology results that were reported in the studies. The Agency, however, based on its understanding of relevant scientific literature, could not conclude that these measurement endpoints are biologically relevant indicators of effects on growth or reproductive success (*i.e.,* the Agency did not interpret these responses as adverse effects *per se*) nor was the Agency aware of any information that established these responses as precursors to the apical endpoints of primary interest (*i..e*., time to and size at metamorphosis, sex ratio, and the presence of mixed and/or intersex animals).

(a) Please comment on the biological relevancy of these endpoints and the extent to which they may reflect reliable measures of developmental abnormalities.

8) The Agency's analysis of potential developmental effects in studies responsive to the DCI has focused on histological data as opposed to gross morphological data. The histological data from these studies are based on the analyses of a single certified pathologist. While this approach eliminates the potential variability associated with having multiple pathologists analyze the histological slides, it may introduce an avidity bias.

(**a)** Please comment on whether a single pathologist is sufficient for interpreting the histology data. If the SAP believes that a single pathologist is not sufficient, please comment on the potential value of convening a pathology review board to evaluate the findings of the DCI study.

(b) Please also comment on the potential value of having a pathology review board evaluate materials (*e.g*., digital images of gross morphology and microscope slides containing histological sections) from studies published in the open literature. These data could be submitted voluntarily by the authors and could include slides to evaluate similarities or differences in identifying or describing histological features and/or describing and quantifying histological responses.

9) After an evaluation of the laboratory-based studies submitted in response to the DCI, the Agency has concluded that these studies do not provide sufficient evidence to support the hypothesis that atrazine causes adverse gonadal developmental effects in amphibians.

(a) In light of the responses to Questions 3 – 8, please comment on whether the results from the study in response to the DCI are sufficiently robust to address the hypothesis that atrazine exposure causes gonadal abnormalities in *X. laevis*. If the SAP concludes these results are not sufficiently robust, what recommendations can the SAP provide to efficiently and reasonably address remaining uncertainties? For example, if the SAP does not believe the DCI study is sufficiently robust to assess the hypothesis, does the SAP believe either the two experiments or a specific component of the two experiments should be reanalyzed or repeated? Please provide the rationale for recommending any additional analyses and/or experiments.

(b) Please comment and provide recommendations on alternate statistical analyses, if any, to evaluate the data derived from the study. If alternative approaches are suggested, please comment, to the extent possible, on the rationale for these approaches and how they represent improvements in the existing statistical interpretations.

Concluding Questions

10) Is the SAP aware of any other laboratory-based or field-based studies not included in this White Paper that contradict the Agency's conclusions that 1) the designs associated with current studies available in the open literature are not appropriate for evaluating the hypothesis that atrazine affects amphibian gonadal development and 2) the available data in the open literature combined with the results of DCI study indicate that atrazine does not cause adverse effects on gonadal development in *X. laevis* when investigated under conditions consistent with those recommended by the SAP in its previous report (SAP 2003). If so, please identify the studies and briefly outline how the results from these studies would contradict the conclusion that atrazine at concentrations up to 100 µg/L does not cause adverse effects on amphibian gonadal development.

11) The Agency is not aware of data that establish a mechanistic basis for how atrazine could affect amphibian gonadal development. Please identify and comment on any studies that demonstrate the mechanistic steps by which amphibian gonadal development could be affected by atrazine, and thereby contradict the Agency's overall conclusions based on

the studies evaluated for this SAP review. If the SAP is aware of any relevant study(ies), please comment on the data from this study(ies) and how the data indicate and quantify a mechanistic pathway from atrazine's molecular site of action to histological and apical endpoints associated with adverse effects on amphibian gonadal development. Please also comment on any dose-response relationships associated with the steps in the reported toxicity pathway.

12) In its 2003 White Paper the Agency proposed a research approach using focused, empirical laboratory studies based on initial investigations with *X. laevis,* potentially followed by selective, confirmatory laboratory studies with frog species native to North America. However, the 2003 SAP did not identify any important differences between amphibian species to conclude that any affected developmental and/or mechanistic processes observed in *X. laevis* would not be applicable to indigenous ranid species.

(a) Please comment on the Agency's recommendation that data derived from *X. laevis* in the studies evaluated for this review are sufficient to conclude that additional testing with indigenous species is not warranted.

13) Based on the available data provided by the DCI studies, the Agency has concluded that atrazine does not adversely affect amphibian gonadal development. The Agency has further concluded that no additional studies are required to address the hypothesis that atrazine adversely affects amphibian gonadal development.

a) Please comment on the Agency's recommendation that the current body of data is sufficient to refute the hypothesis that atrazine by itself can adversely affect amphibian gonadal development and that no additional data are required to address this hypothesis.

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In this study, green frog eggs were collected from a rural pond in Michigan and the eggs were reared in filtered UV-exposed well water. At roughly 5 days post-hatch, larval frogs (Gosner Stage 25) were divided into 10-L tanks containing 4 L of test solution. Each exposure was replicated 9 times and each replicate contained roughly 30 tadpoles. Tanks were maintained in an environmental chamber where temperature, humidity, and light cycle were controlled. Exposures consisted of water alone, 0.005% ethanol, estradiol (0.1 mg/L), dihydroxytestosterone (0.1 mg/L) and 10 or 20 µg/L atrazine. Exposure consisted of static renewal, and test solutions were renewed by 50% every 72 hours. After 67 d of exposure, ammonia nitrogen and nitrite levels rose to "unacceptable levels", and the tadpoles were transferred to 20-L tanks containing 16 L of test solution. After 273 d of static renewal exposure, tadpoles were transferred to a continuous flow-through system of clean fresh water until metamorphosis occurred. At initiation of metamorphosis (forelimb emergence at Gosner Stage 42), tadpoles were removed from the flow-through system and housed individually in 10-L glass tanks, containing 500 mL of fresh water; tanks were tilted so that a wet and dry area were available to the developing animals. After 506 days, the study was terminated and tadpoles that had not reached metamorphosis were sacrificed.

Water samples were collected following each test solution renewal (72-hr intervals), and atrazine concentrations were determined using enzyme-link immunosorbant assay (ELISA) with a method detection limit of 0.074 µg/L and a limit of quantification of 0.22 µg/L. When atrazine concentrations deviated from nominal values, concentrations were verified using gas chromatography/mass spectroscopy.

Mortality, number of frogs initiating metamorphosis, and number of frogs completing metamorphosis were recorded daily. At completion of metamorphosis, juveniles were euthanized with tricaine methylsulfonate, weighed and measured for snout-vent length. Frogs were then preserved in Bouin's solution. Fixed frogs were examined for gross gonadal morphology. A

subset of frogs examined for gross morphology was also examined for histology (serial sections at 5 µm intervals with roughly every 20 µm section analyzed).

According to the authors, measured atrazine concentrations were similar to nominal values. Low concentrations of atrazine were reportedly detected in control exposure water. However, less than 30% of the samples collected from control tanks had atrazine concentrations above the method detection limit of 0.074 µg/L, and less than 17% had atrazine concentrations above the limit of quantification (0.22 µg/L). GC/MS verification of ELISA confirmed atrazine in the control, 10, and 25 µg/L treatments at concentrations ranging between <0.1 – 0.39, 15-29, and 30 – 46 µg/L, respectively.

Under static conditions, water temperature averaged 21.3±1.4 ^{o}C, and total ammonia and nitrite nitrogen averaged 0.04±0.0 and 0.22±0.4 mg/L, respectively; dissolved oxygen averaged 8.0±0.7 mg/L. In the flow-through system, temperature, total ammonia, and nitrite nitrogen, and dissolved oxygen averaged 24.8±0.5^{o}C, 0.02±0.0 mg/L, 0.02±0.0 mg/L and 6.1±1.7 mg/L, respectively. Water hardness was roughly 425 mg/L as $CaCO_3$.

Mortality was reported greatest during the first month of exposure and tended to decrease as the tadpoles aged. Once the tadpoles were transferred to a flow-through system on d 273, mortality decreased. There were no significant differences in mortality between controls and the atrazine-treated group. Mortality was not statistically different between the dihydroxytestosterone, estradiol, and the solvent control although there were fewer deaths in the dihydroxytestosterone treatment (63%) compared to the estradiol (86%) (**Table 25**).

Table 25. Average percent mortality.

Treatment	Initial Sample Size	Average Percent Mortality
Control	285	79.2
Solvent Control	280	74.8
Dihydroxytestosterone	291	62.7
Estradiol	282	85.7
10 µg/L atrazine	292	73.1
25 µg/L atrazine	292	83.3

The first incidence of completion of metamorphosis occurred on exposure Day 112, and by exposure Day 143, 10 tadpoles had completed metamorphosis. Between days 143 and 285, no tadpoles initiated metamorphosis; however, an increased rate of development was observed after exposure day 285. Age at initiation of metamorphosis and completion of metamorphosis was

significantly different between the atrazine-treated groups and the controls; frogs in the 10 µg/L atrazine treatment initiated and completed metamorphosis at a significantly ($p<0.05$) older age compared to both untreated controls and frogs exposed to 25 µg/L atrazine (**Table 26**). Frogs in the estradiol treatment initiated metamorphosis at a younger age compared to both the dihydroxytestosterone and the solvent control animals. The authors suggested that the significantly longer time required for frogs to metamorphose in the 10 µg/L atrazine group was not concentration-dependent since the 25 µg/L atrazine treatment did not exhibit the effect. The authors also noted that the time to metamorphosis and lower weight was apparently not related to an estrogenic effect since estradiol-treated animals exhibited an opposite response.

Frogs in the 10 µg/L atrazine group were significantly shorter (SVL) than frogs in the 25 µg/L atrazine group but were not significantly shorter than controls (**Table 26**). There was no significant difference in body weight.

Table 26. Numbers, mean ages at initiation and completion of metamorphosis, snout-vent lengths and weights at metamorphosis. Standard errors in parentheses.

Treatment	Mean Age at Initiation in Days N	Mean Age at Completion in Days N	Mean Weight (g)	Mean SVL (cm)
Control	328 (8.5) 58	337 (10) 44	1.76 (0.05)	2.52 (0.08)
Solvent Control	350 (7.4) 69	360 (9.5) 47	1.56 (0.04)	2.47 (0.06)
Dihydroxytestosterone	350 (7.1) 104	369 (7.9 75	1.50 (0.03)	2.42 (0.05)
Estradiol	330 (4.8)** 40	342 (4.6) 33	1.64 (0.09)	2.57 (0.06)
10 µg/L atrazine	362 (6.5)* 77	377 (7.3)* 64	1.48 (0.03)	2.39 (0.04)*
25 µg/L atrazine	335 (7.7) 48	342 (7.9) 37	1.64 (0.05)	2.54 (0.07)

*significantly different than negative control ($p<0.05$)
** significantly different than ethanol control ($p<0.05$)

There was no difference in sex ratio between atrazine-treated and control animals (**Table 27**). There was no difference between estradiol-treated and solvent control animals for sex ratio; however, animals treated with dihydroxytestosterone (97.7% males) were significantly different than the solvent control.

Table 27. Sex ratios (percentages) of surviving juvenile frogs.

Treatment	N	Males	Females
Control	51	43.1	56.9
Solvent Control	57	47.4	50.9
Dihydroxytestosterone	86	97.7*	2.3*
Estradiol	38	36.8	63.2
10 µg/L atrazine	72	40.3	59.7
25 µg/L atrazine	44	40.9	59.1

*significantly different than negative control ($p<0.05$)

The incidence of gonadal deformities ranged from 0 to 5.9% across all treatment groups and there were no differences across treatment groups for gonadal anomalies; differential paired gonad size was the most frequent observation. No hermaphroditic gonads (testicular and ovarian tissue in one individual) were identified through gross morphology. Histology did reveal rudimentary hermaphroditism (intersex and testicular oocytes) across all treatments, ranging between 0 to 12.9% with most anomalies occurring in male frogs. Based on histology, testicular oocytes were the most common anomaly in the estradiol-treated animals, solvent control, and control animals; however, testicular oocytes were not observed in atrazine-treated and dihydroxytestosterone animals. There were no statistically significant differences for the incidence of histological anomalies across treatments.

Analysis

This is one of the studies previously reviewed (USEPA 2003) as an interim report (Hecker *et al*. 2003a; MRID 458677-03). The loading rate of 30 tadpoles/4 L exceeds the EPA recommended rate of 1 tadpole per liter. With only 50% of the exposure solutions changed every 72 hours, it is likely that the water quality in the exposure tanks was poor. The authors indicate that by day 67 of exposure, water quality had deteriorated to the point that increased exposure tank sizes were required. The report does not mention what the tadpoles were fed during exposure or whether the exposure tanks were aerated.

Atrazine was detected in the controls repeatedly throughout the study, and concentrations in at least 17% of the control samples exceeded 0.22 µg/L and reached a concentration as high as 0.39 µg/L. Measured concentrations within the 10 µg/L atrazine treatment ranged between 10 to 29 µg/L, while the 25 µg/L treatment ranged between 25 – 46 µg/L. These numbers suggest that measured concentrations varied by as much as 190% for the low atrazine treatment and by as much as 84% for the high atrazine treatments and that there were situations where concentrations in the two treatment groups were similar. In addition, the control tanks contained atrazine, which

the authors attributed to cross reactivity with some constituents [other than atrazine] in the ELISA assay; however, the atrazine detections were confirmed by GC/MS.

Although the loading rates used in the study are considered high, dissolved oxygen concentrations were close to saturation in the static system; however, the flow-through system had lower dissolved oxygen. This difference in dissolved oxygen is surprising since the number of animals per liter per day would have been significantly reduced in the flow-though system.

Mortality was excessive in all of the treatments (> 60%). In the controls, mortality averaged 79%. The highest level of mortality, roughly 86%, was measured in the estradiol-treated animals.

Approximately 10 of the test animals completed metamorphosis by Day 143 of exposure, and no tadpoles even initiated metamorphosis until after day 285, two days after the animals were transferred from static tanks to flow-through tanks. The pause in metamorphosis by the remaining study animals is unusual. These data strongly suggest that culturing conditions prior to transferring the animals to a flow-through system did not favor development of the larvae. The study authors suggest, though, that the resumption of metamorphosis was attributed to the onset of Spring. Since the animals were housed indoors in an environmental chamber with controlled light and temperature, this explanation does not seem plausible.

The relevancy of the statistically significant difference in time to metamorphosis and body length at metamorphosis for frogs treated with 10 µg/L atrazine is unclear and may have been an artifact of the higher sample size for the lower atrazine exposure group. Typically, frogs that require longer to metamorphose weigh more since they would spend more time feeding prior to metamorphosis. However, in this study, the animals that took the longest to initiate and complete metamorphosis were also the smallest. The animals that took the least amount of time to initiate and complete metamorphosis, i.e., the control animals, also happen to have the highest mean body weight. These data suggest that the culture conditions may have been a confounding factor in the growth of the frogs.

In this study, the sample size numbers reported for sex ratios (**Table 27**) are not consistent with the sample sizes reported for animals completing metamorphosis (**Table 26**). In addition, the lack of a significant difference between estradiol-treated animals and the solvent

control suggests that the positive control was not effective since estradiol should feminize males. It is unclear why dihydroxytestosterone was more effective than estradiol at altering sex ratios. While the authors noted that the high ratio of males in dihydroxytestosterone-treated animals indicated that the study provided sufficient exposure times to measure the effects of exogenous chemicals on green frog development, they didn't explain the absence of a significant effect by estradiol on the ratio of females. Because the positive control for an estrogenic effect in this study did not produce a statistically significant effect, this result is particularly problematic for determining whether atrazine can feminize amphibians.

Another significant problem was the need to shift exposure conditions repeatedly throughout the study. Shifting from static renewal to flow-through conditions would likely change the exposure concentration of atrazine. Additionally, when animals were placed in fresh water (presumably without atrazine) after 273 days of exposure, the animals would have differential exposure to atrazine through their development cycle.

In conclusion, there are several factors that severely confound this study, including the widely fluctuating levels of atrazine in the exposure tanks and the presence of atrazine in the controls. The study is further confounded by the very high mortality rates across all exposures groups and the lack of a significant response to one of the two positive controls.

Coady, K. K., M. B. Murphy, D. L. Villeneuve, M. Hecker, P. D. Jones, J. A. Carr, K. R. Solomon, E. E. Smith, G. Van Der Kraak, R. J. Kendall, and J. P. Giesy. 2005. Effects of atrazine on metamorphosis, growth, laryngeal and gonadal development, aromatase activity, and sex steroid concentrations in *Xenopus laevis*. *Ecotoxicology and Environmental Safety* 62: 160 – 173.

In this study, atrazine (97.1%) was dissolved in Frog Embryo Teratogenesis Assay - Xenopus (FETAX) medium. Positive controls (estradiol [98% purity] and dihydroxytestosterone [purity not stated]) were dissolved in ethanol (100%).

Sexually mature adults were obtained from Xenopus Express and were induced to breed. Fertilized eggs were collected and dejellied in 2% L-cysteine in FETAX. Exposure began 72-hrs post-hatch larvae; exposures were conducted in 10-L glass tanks that each contained 4-L of test solution and 30 tadpoles. Treatment concentrations consisted of 0, 0.1, 1.0, 10, or 25 µg/L atrazine, 0.005% ethanol, or 0.1 mg/L estradiol or dihydroxytestosterone with each treatment replicated 8 times. Test solutions were renewed by 50% every 72 hrs. Atrazine concentrations

were tested at time of renewal in both the exposure chambers and in the stock solutions. Mortality and number of larvae completing metamorphosis were recorded daily. At forelimb emergence (Nieuwkoop Faber stage 58), larvae were sequestered using glass dividers to monitor metamorphic stages. At completion of metamorphosis (NF Stage 66), time to initiate and complete metamorphosis, snout-vent length, and weight data were recorded. A subset of NF Stage 66 animals were euthanized using tricaine methylsulfonate (MS-222) to assess gross gonadal morphology and for histological analysis of the gonad and larynx. The remaining juveniles were reared in 4 L of test solution until approximately 1 month post-metamorphosis. Then they were transferred to 40 L aquaria containing 10 L of test solutions, and exposure continued until 2 – 3 months post-metamorphosis. Total exposure time was 185 days.

After 2 – 3 months post-metamorphosis, half the juveniles were euthanized in MS-222 and fixed for histological analysis. The remaining frogs were sampled for blood and dissected for brain and gonad tissue to determine aromatase activity, while blood plasma was used to measure sex steroid concentrations. Blood was collected within a 3-hr time window from 8:30 – 11:30 a.m. spanning 14 days. During this time, one individual from each replicate was randomly selected and sampled each day. Blood samples were kept on ice for several hours, then centrifuged, and the plasma frozen at -80^{o}C. Concentrations of testosterone and estradiol in plasma were measured by competitive ELISA was described by Cuisset *et al.* (1994)[2]; the antiserum for estradiol was reported to cross-react with other steroids by as much as 17%. The range of quantification for the estradiol and testosterone ELISA assays were 0.78 – 800 pg/well. The ^{3}H-labeled water release assay was used to measure aromatase activity in the brain and gonad tissue; protein levels were quantified using the method described by Bradford 1976[3].

Measured concentrations of atrazine in the atrazine test solutions were relatively close to nominal (**Table 28**). Average mortality in the controls was 11.3% and was 2.04%, 11.5%, 18.7%, and 20.3% in the 0.1, 1.0, 10, and 25 µg/L atrazine treatments, respectively. Average mortality in the solvent control, dihydroxytestosterone, and estradiol treatments were 14.3%, 21.3%, and 8.3%, respectively. Average total mortality across all treatments was 16.1%; there was no statistical difference for mortality across treatments (p=0.298). Of the surviving tadpoles,

2 Cuisset, B., P. Pradelles, D. E. Kime, E. R. Kühn, P. Barbin, S. Davail, F. LeMenn, 1994. Enzyme immunoassay for 11-ketotestosterone using acetylcholinesterase as laberl: application to the measurement of 11-ketotestosterone in plasma of Siberian sturgeon. Comp. Biochem. Physiol. 108 (2): 229 – 241.

3 Bradford, M. 1976. A rapid and sensitive mehtod for the quantitation of microgram quantieis of protein utilizing the principle of protein-dye binding. Anal. Biochem 72: 248 – 254.

98.9% initiated metamorphosis; and there was no statistical difference in the age at completion of metamorphosis among treatment groups (p=0.986). The average age at metamorphosis was 72.8 ± 0.04 days (mean ± std error) with no difference in SVL between treatments (p=0.066). However, in a pair-wise comparison of control and solvent control frogs, the ethanol control frogs (1.89 ± 0.01 cm) were longer than negative control frogs (1.75 ± 0.01 cm) (p=0.032). There was no significant difference in weight of the frogs at completion of metamorphosis (p=0.22); however, pairwise comparisons of negative and solvent control frogs indicated that solvent control frogs (0.85 ± 0.02 g) were heavier than negative control frogs (0.70 ± 0.02 g).

Table 28. Nominal and measured concentrations of atrazine in test solutions measured by ELISA.

Treatment	Nominal Concentration of Atrazine in µg/L	Range of Average Measured Concentrations of Atrazine in µg/L (95% confidence interval)
Control	0	0.1 – 0.26 (0.05 – 0.29)
Solvent Control (ethanol)	0	0.0 – 0.22 (0.0 – 0.22)
Dihydroxytestosterone	0	0.0 – 0.22 (0.0 – 0.22)
Estradiol	0	0.0 – 0.22 (0.0 – 0.22)
0.1 µg/L atrazine	0.1	0.22 (0.17 – 0.26)
1.0 µg/L atrazine	1.0	1.0 (0.93 – 1.1)
10 µg/L atrazine	10	16.4 ((14.1 – 18.9)
25 µg/L atrazine	25	28.9 (24.2 – 35.1)

The most commonly occurring gonadal anomaly in Stage 66 (**Table 29**) and 2- to 3-month old juvenile frogs (**Table 30**) was discontinuous gonads; however there was no statistically significant difference in the incidence of gross gonadal anomalies among NF Stage 66 frogs nor among 2- to 3-month old juveniles between treatments.

Table 29. Percentages of gross gonadal morphologies in NF-66 frogs.

Treatment	N	Discontinuous Gonad %	Rudimentary Hermaphrodites		Size irregularities %	Other Anomalies %
			Mixed Sex %	Intersex %		
Control	45	2.2	0.0	0.0	2.2	2.2
Solvent Control	45	0.0	0.0	0.0	0.0	2.2
DHT	42	4.8	2.4	0.0	2.4	4.8
Estradiol	46	6.5	4.4	2.2	0.0	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	0.0	2.2	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 30. Percentages of gross gonadal morphologies in 2- to 3-month old juvenile frogs (n/r=not reported).

Treatment	N	Discontinuous Gonad %	Rudimentary Hermaphrodites		Size irregularities %	Other Anomalies %
			Mixed Sex %	Intersex %		
Control	75	1.4	0.0	n/r	2.7	n/r
Solvent Control	75	2.7	0.0	n/r	1.3	n/r
DHT	72	1.4	0.0	n/r	0.0	n/r
Estradiol	77	2.6	0.0	n/r	0.0	n/r
0.1 µg/L	71	4.2	0.0	n/r	0.0	n/r
1.0 µg/L	79	1.3	0.0	n/r	2.5	n/r
10 µg/L	73	4.1	2.7	n/r	0.0	n/r
25 µg/L	67	3.0	0.0	n/r	0.0	n/r

At a histological level, rudimentary hermaphroditism was only observed in 4 NF Stage 66 frogs from the estradiol treatment group. One "control" frog had unidentified tissue surrounding the testes. In frogs exposed 2 – 3 months post-metamorphosis, testicular oocytes occurred in male frogs from all treatments, while intersex gonads were observed in male frogs from the negative and positive controls, and the 0.1 and 1.0 µg/L atrazine treatment groups (**Table 31**). According to the authors, only 1 to 2 oocytes were present in the entire testes except in the estradiol and 10 µg/L treatments, which had greater numbers of oocytes mixed with testicular tissue. Most of the anomalies in female frogs were small or underdeveloped ovaries; two frogs (8%) in the estradiol treatment were classified as females containing testicular tissue in their gonads and were considered to be feminized males. There was no statistical difference in the incidence of gonadal anomalies between atrazine-treated and control frogs. There was a statistical difference in the

occurrence of testicular oocytes between dihydroxytestosterone and solvent control frogs (p=0.039) with significantly fewer oocytes in dihydroxytestosterone-treated frogs.

Table 31. Gonadal anomalies in male frogs based on histology 2 -3 months post-metamorphosis.

Treatment	N	Rudimentary Hermaphrodites		Other Anomalies
		Mixed Sex %	Intersex %	
Control	25	8.0	16.0	0.0
Solvent Control	25	20.0	4.0	0.0
DHT	25	4.0	0.0	0.0
Estradiol	25	32.0	0.0	0.0
0.1 µg/L	25	12.0	4.0	0.0
1.0 µg/L	25	8.0	0.0	0.0
10 µg/L	25	12.0	0.0	0.0
25 µg/L	25	8.0	0.0	0.0

Actual data on sex ratios were not provided in the study. Rather the authors state that there were no consistent deviations from the expected ratio of 50:50; there was no significant difference in the percentages of females or the percentage of males among control and atrazine-treated frogs. Only one of the estradiol exposed tanks had a skewed sex ratio with a higher percentage of females; there was no difference in the ratios among dihydroxytestosterone, estradiol, or solvent control animals.

The cross-sectional area of the laryngeal dilator muscles from males frogs was significantly larger than that of females (p=0.0001); however, there was no statistical difference between atrazine-treated animals and controls (p=0.476). There was a significant difference (p=0.008) in male laryngeal muscle area between solvent control, dihydroxytestosterone, and estradiol-treated animals with large cross-sectional areas in frogs treated with dihydroxytestosterone. For females, there was no significant difference in cross-sectional area between control and atrazine-treated animals; however, females treated with dihydroxytestosterone had significantly larger laryngeal muscle area (p=0.0001).

Aromatase activities in the gonads of juvenile females (420$\pm$44 fmol/h/mg protein) were significantly (p=0.0001) greater than in males (10.8$\pm$4.03 fmol/h/mg protein); aromatase activity was less than the level of detection in 77% of the males examined. There was no significant difference between controls and atrazine-treated males (p=0.075) nor were there any significant differences between dihydroxytestosterone, estradiol and solvent control males (p=0.382). There was no statistical difference in ovarian aromatase activity between atrazine-treated and control

females. Females exposed to estradiol had significantly (p=0.003) lower aromatase activity than solvent control females.

Brain aromatase levels were statistically different (p=0.024) between male (0.73+0.05 pmol/h/mg) and females (0.90+0.06 pmol/h/mg); however, there were no statistical differences between atrazine-treated and control animals. Estradiol-treated animals had greater brain aromatase activity compared to dihydroxytestosterone-treated frogs (p=0.012). For females, there was no statistical difference between atrazine and untreated controls (p=0.885), nor were there significant differences between dihydroxytestosterone, estradiol, or solvent control females.

Recoveries of estradiol and testosterone ranged between 27 – 107%; the average percent recovery of the hormones in plasma was 76.8%. Estradiol concentrations in both male and female were sometimes below the limit of detection. Juvenile female estradiol levels (40.8$\pm$20.2 ng/mL) were significantly (p=0.02) greater than male estradiol concentrations (27.0$\pm$9.40 ng/mL). Male frogs exposed to 1.0 µg/L had significantly (p=0.001) lower concentrations of estradiol than controls, 0.1 µg/L or 25 µg/L atrazine-treated frogs; plasma estradiol concentrations in males exposed to estradiol were significantly greater than the solvent control males (p=0.008). There was no difference in plasma estradiol concentrations between control and atrazine-treated females nor were there differences between dihydroxytestosterone, estradiol and solvent control females.

This study showed there were no significant differences (p=0.170) in plasma testosterone between males and females; there were no significant differences between male plasma testosterone in atrazine-treated and control frogs (p=0.270); and there was no significant difference in male plasma testosterone between the dihydroxytestosterone, estradiol, and solvent control frogs (p=0.187). Similarly there was no statistical difference between female plasma testosterone in atrazine-treated and control frogs (p=0.179) nor between dihydroxytestosterone, estradiol, and solvent control females (p=0.363). Estradiol and testosterone levels were positively correlated (r^2=0.81), but neither were correlated with either gonad or brain aromatase activity.

Analysis:

This is one of the studies previously reviewed (USEPA 2003) as an interim report (Hecker *et al*. 2003b; MRID 458677-04). As noted previously, the loading rate (30 tadpoles/4 L) exceeds EPA's recommended rate of 1 tadpole/L/day. This high loading rate combined with only 50% fresh water renewal would likely result in poor water quality. Some important method information is missing from the study description such as the number of NF Stage 66 animals that were sub-sampled for histological analysis, the feeding regime for test animals, and the blood collection method. While the researchers attempted to collect blood over a limited amount of time, the collections extended over 4 hours each day, and blood was allowed to sit [on ice] for several hours prior to centrifugation.

Atrazine was reported in all of the treatment solutions, including the solvent, negative controls, and positive controls. The levels detected in controls were within the limit of quantification (0.22 µg/L) for the ELISA assay and within the 95% confidence intervals for the lowest atrazine treatment (0.17 – 0.26 µg/L).

Statistically significant effects on mean length and weight of frogs between solvent and negative controls indicate that the solvent had an effect. It is not clear from the paper whether similar concentrations of solvent were used in each of the controls; however, the solvent effect confounds the study.

In this study, the length of time to metamorphosis (72.8 days) is longer than what is typically seen for *X. laevis* (54 days) and may reflect inadequate rearing conditions. Although positive controls were run, the results from the positive controls do not suggest that they had much of an effect on any of the indices measured in the study. Either the positive controls were not suitable "controls" for the indices measured, or environmental factors in the study may have confounded the results. Confounding effects would include high loading rates, which could have a potential impact on development, and the presence of atrazine in all of the controls. Based on gonadal histology, the authors did not report any statistical difference between estradiol-treated frogs and controls although there was a significant difference between the solvent control and dihydroxytestosterone-treated frogs. These results seem counterintuitive. Although there is only limited information provided on sex ratios, only 1 of the 8 estradiol tanks exhibited a skewed sex ratio in favor of females, suggesting that estradiol was not an effective positive control.

(Estradiol treatment is expected to feminize males.) Of the positive controls and indices measured, only laryngeal dilator muscle area appeared to be responsive to dihydroxytestosterone treatment in female frogs.

It is unclear why gross morphology data were not broken out by sex; however, the results suggest that gross morphology was not a reliable means of detecting gonadal anomalies. For the frogs evaluated after 2-3 months exposure, gross morphology indicated that the incidence of mixed sex ranged between 0 to 2.7%; however, based on histology in males, the incidence of mixed sex ranged between 4 – 20%.

Although testosterone and estradiol values were corrected for recovery, the range (27 – 107%) of apparent recoveries does not appear to be reflected adequately in the 76.8%, which was presumably used to adjust the values.

It is not clear from the paper whether ethanol was added to the atrazine-treatments as well or only to the positive controls and solvent control; however, the data suggest that on length and weight, the solvent control was significantly different from the negative control, implying a possible solvent effect.

In general, atrazine contamination of the controls and delayed development of the test animals suggest that the ability of the study to measure effects is seriously compromised. High variability in hormone and aromatase levels may have resulted from the protracted period of time in which the samples were collected and the delay in processing the samples. Coefficients of variation ranged as high as 95% for plasma hormone levels. Additionally, using median values rather than mean values for plasma hormone levels suggests that the data are not normally distributed but rather are highly skewed with means heavily influenced by extreme values. The lack of a significant effect on sex ratio across all of the positive estradiol controls further supports the inability of the study to demonstrate a measurable effect.

Murphy, M. B., M. Hecker, K K. Coady, A. R. Tompsett, P. D. Jones, L. H. Du Preez, K. R. Solomon, J. A. Carr, E. E. Smith, R. J. Kendall, G. van der Kraak, and J. P. Giesy. 2005. Sediment TCFF-Eq's and EROD and MROD activities in Ranid frogs from agricultural and non-agricultural sites in Michigan (USA). Arch. Env. Cont. Tox. 51(3): 467 – 477.

In this study, corn growing (agricultural) and nonagricultural sites near Kalamazoo and Lansing, MI were sampled twice during summer 2003, and sites were selected based on atrazine concentrations. Atrazine concentrations were based on monthly samples collected from May through September and relied on a triazine ELISA assay with a limit of detection of 0.05 µg atrazine/L and a limit of quantification of 0.17 µg atrazine/L.

Adult green frogs (*Rana clamitans*), bullfrogs (*R. catesbeiana*), and leopard frogs (*R. pipiens*) were collected at night in May, and juvenile frogs were collected at night in July. Frogs were euthanized with methyl tricaine sulfonate, and their livers were flash-frozen in liquid nitrogen.

Microsomes were prepared from 0.5 g adult liver samples, and 7-ethoxyresorufulin *O*-deethylase (EROD) and 7-methoxyresorufin *O*-deethylase (MROD) activities were measured using methods described by Kennedy and Jones (1994) with modifications. Because livers from juvenile frogs weighed less than 0.5 g, the entire liver was used. The optimum incubation temperature for measuring EROD and MROD activity was determined to be 30^{o}C for all species. Where enzyme activities could not be detected, the value was set to one half the lowest detectable EROD and MROC enzyme activity level of 1.46 and 0.84 pmol/min/mg protein in juveniles, respectively.

Sediment (20 g) was extracted, and the extract was removed for use in cell bioassays. Rat hepatoma cells (H4IIE-luc) that express the AhR receptor activity were used in the cell bioassays.

Atrazine concentrations in nonagricultural sites were less than the LOQ (0.17 µg/L) except for one site sampled in August 2003 (0.23 µg/L). Atrazine concentrations at agricultural sites ranged from <0.17 to 1.17 µg/L. Only two of the sample sites exhibited atrazine concentrations with levels $\geq$0.45 µg/L.

Sediment TCDD-EQs were <1 pg TCDD-EQ /g dry weight at all sites with the exception of one agricultural site (Ag2). Dioxin-like activity, when measurable, was observed in the second and third Florasil column fractions of extracted sediments.

Liver-somatic indices (LSI) were comparable between adult and juvenile green frogs, but were overall greater in adult female frogs than in male or in male or female juvenile green frogs. For juvenile green frogs, LSI was significantly different between sites but did not differ significantly between agricultural and nonagricultural sites. For adult green frogs, only male adult frogs showed a significant difference between agricultural and nonagricultural sites.

For EROD enzyme activity, there was no significant difference between agricultural and nonagricultural sites for either adult or juvenile green frogs for either sex. MROD activity was 2- to 3-times lower than EROD activity. MROD activity was less than the detection limit in 25% of the juvenile female frogs and 30% of the juvenile male frogs. MROD was significantly different ($p<0.001$) for male juveniles between agricultural and nonagricultural sites; MROD activity in male juvenile frogs was significantly greater at agricultural sites.

Median LSI was significantly and positively correlated with both median EROD ($p<0.05$) and MROD ($p<0.025$) in adult female green frogs but not in adult male green frogs. In juvenile male ($p=0.020$) and female ($p=0.002$) green frogs, median LSI was significantly and negatively correlated with median EROD activity.

Median LSI, EROD, and MROD activities were not correlated with atrazine water concentrations measured 4 weeks before sampling or at the time of sampling in adult female green frogs. Median MROD activity was negatively correlated with atrazine concentrations measured 4 weeks prior to sampling in adult male frogs. There were no significant correlations between atrazine concentrations and LSI, EROD, or MROD in either female or male juvenile green frogs.

Only one of the sites (AG2) had measurable TCDD-EQ in the sediment, and this level was not significantly correlated with LSI, EROD, or MROD for either juvenile male or female green frogs. In adult female green frogs, EROD activity was significantly ($p<0.017$) less at AG2 compared with other sites, while no relationship could be demonstrated for adult male frogs.

Although the authors note that atrazine concentrations were significantly and negatively correlated with MROD activity in adult male green frogs, they conclude that atrazine does not appear to have a consistent association with EROD and MROD activities in wild-caught green frogs. Therefore, the authors conclude that the atrazine-mediated EROD induction observed *in vitro* for ranids does not occur in wild ranid frogs collected in Michigan and that it is unlikely that atrazine is affecting EROD and MROD in wild ranids.

Analysis

This is one of the studies previously reviewed (USEPA 2003) as an interim report (Jones *et al*. 2003; MRID 458677-02). Sample selection was based on atrazine concentrations and the lack of corn production; however, monitoring data to determine the presence or absence of atrazine and/or N-triazine degradates may not have been sufficient to fully document the presence and/or absence of the chemical(s). Chemical monitoring only looked at atrazine, and the potential exposure to other chemicals is unknown. Test animals were collected from the wild, and their previous exposure history to chemicals is not known. While the paper states that green frogs are territorial and are "highly faithful" to specific breeding ponds and wetland habitats throughout the year, it is not clear if the other species in the study share in this behavior. The authors claim to have differentiated young-of-the-year tadpoles from those that may have over-wintered as tadpoles (1-yr old tadpoles) based on the size of the tadpole. The reliability of this method, for aging tadpoles, however, is uncertain.

Atrazine concentrations in agricultural ponds varied considerably (<0.17 to 1.17 µg/L) with some agricultural sample sites having measured atrazine concentrations roughly similar to the controls. These measured concentrations of atrazine, though, do not provide any information on common [triazine] degradates that may have provided a better indication of total exposure.

In this study, frogs were collected at night with a target sample size of 40-50 frogs/site/age class and a minimum sample size of 22 animals. It is not clear from the report whether frogs were then maintained alive until they were returned to lab and then euthanized or whether the frogs were immediately euthanized upon capture. If frogs were maintained alive, then the loading rates within the collection vessels would likely change as the night progressed, and it is likely that water quality within the holding tanks changed markedly. The study does not distinguish what constitutes a "juvenile" frog. It is unclear whether this term is used to represent

animals that have just completed metamorphosis or those that simply have no secondary sexual characteristics, which would likely make it difficult to accurately assess the gender of the animal by external appearance. By the same token, no information is provided on the age of the animals or the study animals' stage of development relative to the breeding cycle during the study. These factors may have influenced hepatic enzyme activity.

Since specific liver sample sizes (0.5 g) were required and were flash frozen, it is likely that tissue samples were not processed until they were returned to the laboratory. Since this study involved measuring liver enzyme activity, the sampling process may have affected hepatic enzyme activities and contributed to variability that made it difficult to measure potential differences between sampling sites.

Three different fractions from a Florasil column following extraction of sediments were presumed to contain increasing percentages of polar organic compounds; however, no information is presented on how or whether these assumptions were verified. It appears that spiked sediments were not run to verify whether the sediment TCDD-EQs approach provides consistent results.

Measurement endpoints (GSI, EROD and MROD) for green frogs appeared to be highly variable between sites, and this variability made it difficult to detect any differences between agricultural versus nonagricultural sites. Although not discussed in the paper, the sample sizes reported for the different measurement endpoints indicates that sex ratios were heavily skewed toward male animals. Only two of the ponds (AG3 and AG2Fall) sampled contained similar numbers of male and female green frogs. In one of the control ponds, no females were reportedly collected. It is unclear why the sex ratios were so skewed. The absence of data on sampled female frogs from one of the nonagricultural pond reduces the ability of the test to discriminate effects, reducing the sample size to 2.

Sex ratios for juvenile frogs (**Table 33**) were less skewed in favor of males than were the sex ratios for adult frogs (**Table 32**). The fewer reported number of ponds suggest that juvenile frogs were not well represented. Additionally, the sample size is reduced to 2 because no juvenile frogs were apparently collected from one (AG3) of the agricultural sites.

Table 32. Numbers of male and female adult green frogs sampled for liver somatic index from nonagricultural (NA) and agricultural (AG) sites.

Site	Females	Males	Sex Ratio
NA1	6	16	0.27
NA4	0	13	0.0
NA5	8	36	0.18
AG2	4	21	0.16
AG3	9	7	0.56
AG6	6	16	0.27
AG7	6	22	0.21
AG8	15	26	0.37
AG9	7	36	0.16
AG10	3	24	0.11
AG2 Fall	16	23	0.41

Table 33. Numbers of male and female juvenile green frogs sampled for liver somatic index from nonagricultural (NA) and agricultural (AG) sites.

Site	Females	Males	Ratio
NA1	25	20	0.56
NA4	15	27	0.36
NA5	23	28	0.45
AG2	28	19	0.60
AG6	0	0	0.0
AG9	24	23	0.51

Although the study discusses results from bullfrogs and leopard frogs, the sample sizes across agricultural and nonagricultural sites were insufficient to test for statistical differences.

In general, the study is confounded by highly variable results that make it difficult to detect any statistically significant effect between agricultural and nonagricultural sites. The reported levels of atrazine in the agricultural sites appear to differ considerably among sites. Further limiting the study is the absence of data on atrazine degradates or any other chemical. The lag time between when samples were collected and analyzed may have contributed to the variability in enzyme assays. The discussion of the high variability associated with EROD and MROD activities underscores the possibility that these may not be reliable means of measuring potential effects of chemicals in the field. The authors also note that the TCDD-EQ activity at one of the ponds (AG2) may have been attributed to polyaromatic hydrocarbon (PAH) chemicals in runoff from a nearby road. Finally, the skewed sex ratios suggest that the sampling technique was biased toward males, and it is unclear whether this study could be considered representative of natural populations.

Although the authors note that atrazine concentrations were negatively correlated with MROD activity in adult male green frogs, the biological relevancy of either induction or suppression of MROD activity is uncertain.

Murphy, M. B., M. Hecker, K K. Coady, A. R. Tompsett, P. D. Jones, L. H. Du Preez, G. J. Everson, K. R. Solomon, J. A. Carr, E. E. Smith, R. J. Kendall, G. van der Kraak, and J. P. Giesy. 2006a. Atrazine concentrations, gonadal gross morphology and histology in ranid frogs collected in Michigan agricultural areas. *Aquatic Toxicology* 76: 230 – 245

In this study, corn growing (agricultural) and nonagricultural sites near Kalamazoo and Lansing, MI, were sampled twice during summer 2003. The sites selection was based on atrazine concentrations. Water samples (1 L), which were collected 10 cm below the water's surface, were analyzed for atrazine and other agricultural chemicals. Monthly samples were collected from May through September, and atrazine concentrations were analyzed using an Enviroguard® triazine ELISA assay, with a limit of detection of 0.05 µg atrazine/L and a limit of quantification of 0.17 µg atrazine/L. Aquatic and terrestrial plants were collected at each site to determine dominant and subordinate plant types.

Adult green frogs (*Rana clamitans*), bullfrogs (*R. catesbeiana*), and leopard frogs (*R. pipiens*) were collected at night in September and October 2002 and in May and June of 2003; juvenile frogs were collected at night in July of both years. The target sample size of 40 – 50 frogs per site per age class was established with a minimum of 13 frogs. Animals were visually inspected for external malformations and were euthanized using methyl tricaine sulfonate (MS-222). Gonads were visually inspected for malformations. If normal, the right gonad was removed, weighed, and then flash frozen for aromatase measurements. The remaining gonad was left in place and the animal was fixed in Bouin's solution for 48 hrs. If visual inspection revealed gonadal malformations, both gonads were left in place and fixed

Histology was conducted on male frogs only using serial sections (every 7.5 µm). Sections were stained in hematoxylin and eosin. Testicular oocytes were counted and staged. A male gonad containing >30% testicular oocytes was classified as ovotestes.

In 2002, atrazine concentrations were <0.17 µg/L in nonagricultural sites (NA1, NA2 and NA3), and in 2003, atrazine concentrations at nonagricultural sites ranged from <0.17 – 0.23 µg/L (NA1, NA3 and NA5). At agricultural sites, atrazine concentrations ranged from <0.17 –

250 µg/L in 2002 (AG1, 2, 3, 4, 5a and 5b) and from <0.17 – 0.73 µg/L in 2003 (AG2, 3, 6, 7, 8, 9 and 10).

None of the other agricultural chemicals screened for were detected in 2002 and only arsenic and zinc were observed at concentrations >5 µg/L (LOQ). According to the report, malformations in frogs were observed in 4 of 8 sites in 2002 and 5 out of 10 sites in 2003. However, according to the tabular summary, malformations were observed for adults at 2 sites and for juveniles at 3 (total of 4 sites) of the 8 sites in 2002. In 2003, the tabular summary indicates that 4 out of the 10 sites for adults and 4 (total of 6 sites) out of the 10 sites for juveniles exhibited malformations. Malformations included fused digits (syndactyly), missing digits (ectrodactyly), extra digits (pollydactyly), and missing limbs (ectromelia). Missing eyes were found in one juvenile in 2002 and one adult and one juvenile in 2003. There was no significant difference in the incidence of malformations between agricultural and nonagricultural sites or in adults or juveniles.

Site-specific incidence of hermaphroditism in green frogs was <5% at both agricultural and nonagricultural sites in both years of the study. One juvenile frog from the agricultural sites and one from nonagricultural sites were collected in 2002, while no juvenile hermaphroditic green or leopard frogs were collected in 2003. No hermaphroditic adults were collected in 2002, and in 2003 one adult hermaphroditic green frog was collected from an agricultural site. Discontinuous gonads were found in only one adult green frog from a nonagricultural site in 2002 and in 2 males frogs collected from a nonagricultural site in 2003.

Testicular oocytes were observed in all three species collected both in 2002 and 2003 from most of the ponds, and there was no statistical difference between agricultural and nonagricultural sites in adults ($p>0.995$) or juveniles ($p>0.1$) from 2002 or in adults collected in 2003 ($p>0.995$). However, the incidence of testicular oocytes was significantly greater at agricultural sites in juveniles collected in 2003 ($p<0.005$) and was correlated with maximum atrazine concentrations. The authors state, though, that "given the fact that juveniles were collected at the same time in both years of the study as well as the fact that a greater range of atrazine concentrations was measured in 2002 when no correlation with TO incidence was observed, this inconsistent result makes it unlikely that the TOs found in male frogs were related to atrazine exposure, including the relatively great concentrations measured during at least part of the tadpole development period in 2002."

In the paper's discussion, arsenic and zinc levels were stated to be high, with arsenic levels near the national drinking water standard of 10 µg/L. Zinc levels in some at some of the agricultural sites were as high as 53 µg/L. The effects of elevated metal levels in the water is an uncertainty in this study.

The study concludes that no consistent change in the incidence of gonadal abnormalities was observed at either the gross morphological or histological level in green frogs, leopard frogs, or bullfrogs collected at agricultural and nonagricultural sites. Testicular oocytes (**Tables 34** to **37**) were correlated with exposure to atrazine in juvenile frogs in 2003, but in no other age-class or sampling year. There were, however, marked differences in the incidence of gonadal abnormalities between Ranid species.

Table 34. Sex ratio and number of testicular oocytes (TO) and number of TO per adult frog by species and site in 2002.

Site	Sex ratio (M:F) (ratio of females)	# Frogs with TO	# TO per Animal	Species
NA1	39:10 (0.20)	2	1	*R. clamitans*
NA2	30:15 (0.33)	2	2, 15	*R. clamitans*
NA2	8:11 (0.58)	2	1, 2	*R. pipiens*
NA3	28:18 (0.39)	5	1, 1, 1, 3, 27	*R. clamitans*
AG1	9:4 (0.31)	0	na	*R. catesbeiana*
AG2	13:4 (0.24)	0	na	*R. clamitans*
AG2	14:16 (0.53)	1	95	*R. catesbeiana*
AG3	13:18 (0.58)	2	3, 32	*R. clamitans*
AG3	6:10 (0.63)	0	na	*R. catesbeiana*
AG4	28:22 (0.44)	8	1, 2, 2, 4, 5, 6, 7, 33	*R. clamitans*
AG5	7:9 (0.56)	1	1	*R. clamitans*
AG5	2:1 (0.33)	1	76	*R. catesbeiana*

Table 35. Sex ratio and number of testicular oocytes (TO) and number of TO per juvenile frog by species and site in 2002.

Site	Sex raio (M:F) (ratio of females)	# Frogs with TO	# TO per Animal	Species
NA1	32:23 (0.42)	1	1	*R. clamitans*
NA2	24:12 (0.33)	0	0	*R. clamitans*
NA2	4:1 (0.20)	2	1, 8	*R. pipiens*
NA3	24:23 (0.49)	1	5	*R. clamitans*
AG1	5:11 (0.69)	0	0	*R. clamitans*
AG2	54:40 (0.43)	4	1, 1, 4, 12	*R. clamitans*
AG2	--	--	--	*R. clamitans*
AG3	28:13 (0.32)	4	1, 1, 1, 7	*R. clamitans*
AG3	--	--	--	*R. clamitans*
AG4	23:24 (0.51)	2	1, 11	*R. clamitans*
AG5	14:9 (0.39)	1	1	*R. clamitans*
AG5	--	--	--	*R. clamitans*

Table 36. Sex ratio and number of testicular oocytes (TO) and number of TO per adult frog by species and site in 2003.

Site	Sex ratio (M:F) (ratio of females)	# Frogs with TO	# TO per Animal	Species
NA1	16:6 (0.27)	1	81	*R. clamitans*
NA4	13:1 (0.07)	1	1	*R. clamitans*
NA4	13:15 (0.54)	0	n/a	*R. catesbeiana*
NA5	36:8 (0.18)	3	1, 4, 4	*R. clamitans*
AG2	44:20 (0.31)	3	1, 1, 2	*R. clamitans*
AG2	8:20 (0.71)	0	n/a	*R. catesbeiana*
AG3	7:9 (0.56)	1	1	*R. clamitans*
AG3	6:3 (0.33)	0	n/a	*R. catesbeiana*
AG6	16:6 (0.27)	2	6, 7	*R. clamitans*
AG6	4:5 (0.56)	0	n/a	*R. pipiens*
AG7	26:15 (0.37)	3	1, 1, 1	*R. clamitans*
AG8	36:7 (0.16)	1	2	*R. clamitans*
AG9	24:3 (0.11)	5	1, 1, 2, 3, 5	*R. clamitans*
AG10	22:6 (0.21)	2	1, 5	*R. clamitans*

Table 37. Sex ratio and number of testicular oocytes (TO) and number of TO per juvenile frog by species and site in 2003.

Site	Sex raio (M:F) (ratio of females)	# Frogs with TO	# TO per Animal	Species
NA1	20:25 (0.56)	0	n/a	*R. clamitans*
NA4	27:15 (0.36)	1	3	*R. clamitans*
NA4	--	--	--	
NA5	28:23 (0.45)	3	4, 5, 8	*R. clamitans*
AG2	19:28 (0.60)	3	1	*R. clamitans*
AG2	--	--	--	--
AG3	--	--	--	--
AG3	--	--	--	--
AG6	28:14 (0.33)	22	1, 1, 3, 3, 5, 7, 9, 9, 10, 11, 12, 12, 15, 21, 26, 30, 31, 45, 48, 57, 69, 148	*R. pipiens*
AG6	--	--	--	--
AG7	--	--	--	--
AG8	--	--	--	--
AG9	22:24 (0.52)	6	1, 1, 2, 2, 2, 6	*R. clamitans*
AG10	--	--	--	--

Analysis

Similar to the preceding study, this is one of the studies previously reviewed (USEPA 2003) as an interim report (Jones *et al*. 2003; MRID 458677-02). The study reports that measurable concentrations of atrazine were detected in control ponds. The data also show that different sample sites were reported for the two years. If these sample sites are the same as those sampled in the previous study, the concentrations of atrazine appear to be higher than in the ponds characterized in this study. Previously, the highest concentration measured in the agricultural ponds was 1.17 µg/L; however, in this paper, the highest reported concentration is 250 µg/L.

Similar to the previous study, it is unclear what constitutes a "juvenile" frog. It is also unclear whether this term represents animals that have just completed metamorphosis or those that simply have no secondary sexual characteristics, which could make it difficult to accurately assess the gender of the animal by external appearance. By the same token, the aqre of the adults and their stage in the breeding cycle are bit reported; these factors may have influenced gonadal morphology. Adult frogs were collected in Fall of 2002, while in 2003 they were collected in

Spring. The authors suggest that the wide range of variability in gonadal development exhibited by the frogs may serve to protect them against endocrine disrupting chemicals since not all of the animals would be vulnerable.

The authors also note that previous efforts to document gonadal deformities have been compromised by inconsistent terminology to describe specific effects.

Although the authors note that there was no significant difference in the incidence of malformations between agricultural and nonagricultural sites, there were no malformations recorded among juvenile green frogs collected in 2002. Additionally in 2003, no juvenile green frogs were collected at 5 of 7 agricultural sites.

Too few leopard frogs and bullfrogs were collected in this study to support any statistical analyses for examining potential relationships between atrazine and the collection areas.

Murphy, M. B., M. Hecker, K. K. Coady, A. R. Tompsett, E. B. Higley, P. D. Jones, L. H. Du Preez, K. R. Solomon, J. A. Carr, E. E. Smith, R. J. Kendall, G. Van Der Kraak and J. P. Giesy. 2006b. Plasma steroid hormone concentrations, aromatase activities, and GSI in ranid frogs collected from agricultural and non-agricultural sites in Michigan (USA). *Aquatic Toxicology* 77: 153 – 166.

In this study, sites were selected based on atrazine concentrations in corn-growing (agricultural) and nonagricultural sites near Kalamazoo and Lansing, MI. The sites were sampled twice during summer 2003, and water samples (1 L), which were collected 10 cm below the water's surface, were characterized for atrazine and other agricultural chemicals. Monthly samples were collected from May through September, and atrazine concentrations were analyzed using an Enviroguard® triazine ELISA assay, with a limit of detection of 0.05 µg atrazine/L and a limit of quantification of 0.17 µg atrazine/L.

Adult green frogs (*Rana clamitans*), bullfrogs (*R. catesbeiana*) and leopard frogs (*R. pipiens*) were collected at night in August and September 2002 and in May 2003; adults were also sampled at one site in September 2003. Juvenile frogs were collected at night in July of both years. The target sample size of 40 – 50 frogs per site per age class was established with a minimum of 17 frogs. Animals were euthanized using methyl tricaine sulfonate (MS-222), then weighed, and snout-vent length (SVL) was measured. Blood was collected via cardiac puncture from adults only in 2002 and from adults and juveniles in 2003. Blood was collected in a 4-hr

time window at approximately the same time of day. Gonads were visually inspected and photographed and if deemed to have normal morphology, the right gonad was removed and flash frozen in liquid nitrogen for aromatase determinations. The remaining gonad was left in place and fixed in Bouin's solution for later histological analysis. Gonads that were thought to be abnormal were left in place and were fixed for histology.

Plasma was centrifuged at 10,000 rpm for 15 min at room temperature and then frozen at -80^{o}C. Testosterone (T), estradiol (E2), and ketotestosterone (KT) concentrations were measured using ELISA. Because of the small quantities of plasma available from juvenile animals, only T and E2 were measured in 2003. Aromatase activity was measured using the methods developed by Lephart and Simpson (1991) with modifications. Protein concentrations were determined using the Bradford (1976) assay.

There was a general trend to greater plasma androgen levels and lower plasma E2 concentrations in early summer 2003 when compared to late summer 2002. For adult males in 2002, the gonadosomatic index (GSI=gonad weight ÷ body weight) and the ratios E2/T and KT/T differed significantly between agricultural and nonagricultural sites, with significantly greater values observed at agricultural sites. In 2003 for adult males, T and the ratios E2/T and KT/T differed significantly between agricultural and nonagricultural sites, with T concentrations and KT/T values significantly greater at agricultural sites and E2/T values higher at nonagricultural sites. For juvenile male green frogs collected in 2003, only T concentrations and E2/T ratios were significantly greater in agricultural sites compared to nonagricultural sites. Aromatase activities were less than the lowest measured activity in the assay (0.58 fmol/h/mg protein) in most of the testes analyzed, and thus statistical comparisons were not possible.

For adult females collected in 2002, E2/T, KT-IR (ketotestosterone-immunoreactive), and KT/T values were significantly different in agricultural sites compared to non-agricultural sites, with greater values at agricultural sites. In 2003, none of the parameters differed between agricultural and non-agricultural sites. In juvenile female green frogs, only T concentrations differed between agricultural and non-agricultural sites with greater concentrations measured at agricultural sites. Aromatase activity was higher in females than in males but was still less than the LOD in 7 of 136 adult females and 55 of 124 juveniles collected in 2002. The activities in adult and juvenile female green frogs were significantly greater in frogs from agricultural areas.

Similar to the two preceding reviews, this is one of the studies previously reviewed (USEPA 2003) as an interim report (Jones *et al*. 2003; MRID 458677-02). Some of the deficiencies in this study include the following:

- Atrazine concentrations measured approximately 4 weeks before the collection of frogs and at the time of sampling were not correlated with GSI, T, E2, E2/T, KT-IR, KT/T, or aromatase activity in adult male or juvenile female green frogs collected in 2002 or 2003.

- The study methodology does not report how the gonadosomatic index was measured. Were gonads either removed for immediate flash freezing or left in place for fixation and eventual histology?

- ELISA antibodies appeared to cross-react with a number of chemical species beyond what they were intended to measure.

- The authors attribute the variability in plasma hormone concentrations between the two sampling years to seasonal factors. However, the size of the animal, availability of suitable quantities of plasma, and the limit of detection for the various assays can also contribute to the variability. (Low numbers of Northern leopard frogs and bullfrogs prevented statistical comparisons between species.)

Smith, E. E, L. Du Preez, A. Gentiles, K. R. Solomon, B. Tandler, J. A. Carr, G. Van der Kraak, R J. Kendall, J. P. Giesy and T. Gross. 2005. Assessment of laryngeal muscle and testicular cell types in *Xenopus laevis* (Anura Pipidae) inhabiting maize and non-maize growing areas of South Africa. *African Journal of Herpetology*. 54(1): 69 – 76. (MRID 469656-04).

In this study, three maize-growing region (MGR) sites were selected based on the presence of *X. laevis* and the proximity to maize production, use of atrazine and terbuthylazine, and the presence of atrazine in samples taken during the winter preceding the study. Conversely, five nonmaize-growing region (NMGR) sites were selected based on the presence of *X. laevis*, the lack of maize production, and atrazine/terbuthylazine concentrations in water at background concentrations during the winter preceding the study. MGR sites were situated in Viljoenskroon (Free State Province, South Africa), while NMGR were 50 km to the north near Potchefstroom (North West Province, South Africa).

A total of 115 frogs were collected from NMGR sites, and a total of 92 frogs were collected from MGR sites. Frogs (8 – 112 g) were collected in April through May 2002 and held in 2-l plastic containers for 48 hrs, euthanized in tricaine methylsulfonate, and the weights and snout-vent lengths were measured. Gonads and larynxes were dissected and weighed. The left gonad was then fixed for histology, and the other gonad was used to assess aromatase activity. The larynx was fixed.

Three histological sections of the testes (rostral, middle, and caudal) were imaged, and the photomicrographs were used to determine the relative fractional volume of each component (e.g., spermatogonia, spermatocytes, spermatozoa, blood vessels, and connective tissue) and stage of spermatogenesis. Every 12^{th} section was scanned for testicular oocytes.

In this study, the body mass of females was significantly greater than those of males ($p=0.0001$). Female laryngeal cartilage mass, as measured by cartilage mass, was significantly greater than those of males ($p=0.0167$); however, male laryngeal somatic index (LSI) was significantly greater in males than in females ($p<0.0001$). Female length (SVL) was also greater than in males. The mean female SVL from NMGR was 71.8 mm and did not differ significantly from females from MGR (73.9 mm). There was no significant difference in female body weight from NMGR (36.5 g; N=60) and MGR (43.0 g; N=48).

The mean mass of male frogs collected at NMGR (26.1 g) was not statistically different from the mean mass of male frogs collected at MGR (27.6 g). The mean mass of the left testis of frogs collected at MGR (18.6 mg) did not differ significantly (p=0.29) from that collected at NMGR (23.3 mg). Although actual values are not provided, the authors report that mean gonadal somatic index for males collected at MGR did not differ significantly from those collected at NMGR.

Out of 101 male frogs examined, 6 had testicular abnormalities (discontinuous testes or unequal-sized testes) based on gross morphology. No abnormalities were observed in female frog ovaries. According to the authors, testicular histometric analysis did not reveal any statistically significant differences between MGR and NMGR for any of the cell types identified; all of the animals had "normal" appearing testes. The incidence of testicular oocytes at NMGR and MGR was 3% and 2%, respectively.

The paper concludes that there is little evidence for an endocrine-disrupting effect of agricultural chemicals used in maize production and that there is a "small" incidence of Stage 1 testicular oocytes in adult male frogs collected from NMGR (3%) and MGR (2%). Additionally, the authors state that, based on their analysis of testicular cell types, it is unlikely that the low incidence of testicular oocytes resulted in functional sterility or reduced fecundity.

This study was one of the studies previously reviewed (USEPA 2003) as an interim report (Smith *et al*. 2003). The study design is unbalanced with 3 reference (NMGR) sites and 5 MGR sites. The mean mass of female frogs collected from MGR discussed in the text (42.8 g) differs from the data reported in Table 1 (43.0 g). The mean mass of male frogs from both MGR and NMGR reported in Table 1 of the report (*i.e.*, 27.6 and 26.1 g, respectively) differ from those discussed in the text (*i.e.*, 27.0 and 25.8 g, respectively). Based on the sample size reported in Table 1, there were 99 male and 108 female frogs collected in total; however, the text claims that 101 male frogs were examined. The paper does not discuss the levels of atrazine or other triazines/pesticides measured at the sampling sites. Given that triazine concentrations were reported in the reference pond in the interim document, this finding could hold significance for the interpretation of the published results.

Although the tables report sample sizes (N value) for body mass, laryngeal mass, and laryngeal somatic index means, the text reports that the study units considered were the ponds

themselves. Presumably, the means being tested represent treatment means and not individual animal means.

Du Preez, L. H., K. R. Solomon, J. A. Carr, J. P. Giesy, T. S Gross, R. J. Kendall, E. E. Smith, G. Van Der Kraak, And C. Weldon. 2005. Population Structure of the African Clawed Frog (*Xenopus laevis*) in Maize-Growing Areas with Atrazine Application Versus Non-maize-growing Areas in South Africa. *African Journal of Herpetology* 54(1): 61 – 68.

In this study, five maize-growing region (MGR) sites were selected based on the presence of *X. laevis* and the proximity to maize production, previous use of atrazine and terbuthylazine, and the presence of atrazine in samples taken the winter preceding the study. Conversely, for the control sites, three non-maize growing region (NMGR) sites were selected based on the presence of *X. laevis*, the lack of maize production and the absence of detectable atrazine/terbuthylazine residues in water the winter preceding the study. MGR sites were situated in Viljoenskroon (Free State Province, South Africa), while NMGR were 50 km to the north near Potchefstroom (North West Province, South Africa).

Frogs were collected in baited traps. The traps were checked after 48 hours. Animals were transferred to labeled containers and then transferred to the laboratory where the animals were sexed, weighed, and measured for snout-vent length (SVL). Two phalanges of the longest toe on the right forelimb were excised for age determination. Immediately before release, animals were cryogenically branded using liquid nitrogen with a number to indicate their time/site of capture. Mark and recapture population estimates were made using the Jolly-Seber Stochastic method (Donnelley and Guyer 1994). Differences in age distribution between MGA and NMGA were tested using Chi square homogeneity test.

No detectable residues of atrazine were measured in the NMGA ponds. Atrazine concentrations in MGA ranged from 0.12 to 1.23 μg/L. For all the sites, except one of the NMGA and one of the MGA sites, more females than males were collected. Sex ratios across sites were significantly different ($p=0.03$) from a ratio of 50:50; two MGA sites (ratio=34%) and 1 NMGA (ratio=52%) significantly deviated from 50:50. The mean percentage of males for the NMGA and MGA sites was 45% and 42%, respectively.

Male mean body weight ranged from 17 – 29 g and from 14.5 – 21 g at NMGA and MGA, respectively. For females, mean body weight ranged from 35 – 45 g at NMGA and from

21 – 34g at MGA. Log-transformed female body weight was significantly different (p=0.02) between NMGA and MGA. There was no statistical difference between sites for male body weights.

Mean SVL for males ranged from 53 to 63 mm in NMGA and from 50 to 58 mm in MGA. For females, mean SVL ranged from 66 to 72 mm and from 57 to 69 mm in NMGA and MGA, respectively. Average SVL was significantly different. Mean SVL for females was 68 mm and 62 mm for NMGA and MGA, respectively; mean SVL for males was 57 and 52 mm for NMGA and MGA, respectively. Mean SVL was not significantly different between reference and atrazine sites for females (p=0.07) and males (p=0.25).

Eighty-eight percent of the frogs examined were less than 3 years old; the oldest frogs recorded were 6 and 8 years old from NMGA and MGA, respectively. Mean ages differed significantly across all sites (p=0.0001). There was no significant difference in 1, 2, 3 and 4+ age classes between NMGA and MGA; the average age across NMGA and MGA was 1.9 and 2.1 years, respectively, and was not statistically different (p=0.46).

The report concludes that in terms of morphometry, sex ratios, and age class, there were no significant differences between populations of *X. laevis* in MGA and NMGA sites. They attribute the significant difference in female body weights between sites to differential ovarian development since the heavier groups were collected in potentially warmer climates; however, they note that the trend in female body weight appeared to parallel the atrazine concentrations measure at the sites prior to sampling.

The document is related to a nonguideline study that was previously reviewed (USEPA 2003) as an interim report (Smith *et al*. 2003; MRID 458677-10). As discussed previously, it is unclear why frogs were collected in the field, measured in the laboratory and returned to the field since the transit time would have likely stressed the animals and may have affected mortality rates. Population estimates could have been skewed particularly for some collection sites that may have been more distant from the laboratory than others.

The study reports that no atrazine residues were detected in August when frog collection was initiated, the authors do not report whether terbuthylazine or any N-triazine degradates were detected at the reference sites. Additionally, the exposure history of the animals prior to the

monitoring conducted for this study is unknown. Since animals collected during the study were on average roughly 2 years old and at least one was 8 years old, prior exposure to N-triazine herbicides may have occurred among frogs at reference sites. The authors note that it is also possible that frogs from MGA could have migrated to the NMGA and thus their prior exposure history was obscured. Additionally, the authors also note that prolonged prior exposure to pesticides may have selected for more chemically-resistant animals in the MGA and lack of significant differences to populations collected in NMGA results from this selection process. These uncertainties underscore potential confounding effects in trying to distinguish whether field-collected animals actually met the criteria for serving as reference populations.

Although the paper discusses population estimates for the study ponds, the relevancy of this information is uncertain given that the study ponds were not of similar size and had differing habitats.

Jooste, A. M. L. H. Du Preez, J. A. Carr, J. P. Giesy, T. S. Gross, R. J. Kendall, E. E. Smith, G. L. Van Der Kraak and K. R. Solomon. 2005. Gonadal Development of Larval Male *Xenopus laevis* Exposed to Atrazine in Outdoor Mesocosms. *Environmental Science and Technology* 39: 5255 – 5261. (MRID 469656-03).

In this study, twelve outdoor in-ground polyethylene-lined microcosm tanks (2.25 m long x 1.2 m wide x 1.0 m deep) were maintained at North-West University (Potchestroom, South Africa). Each tank was surrounded by a 20-cm high wall to prevent surface water runoff from entering microcosms. The bottom of each tank was covered with a 3-cm layer of sandy soil, and tanks were filled with 1100 L of dechlorinated city water. Water levels were maintained with additional tap water as needed. Macrophytes (*Ceratophylum*) were allowed to colonize the tanks for 5 months prior to study initiation. Atrazine treatments consisted of 1, 10, or 25 µg/L dissolved in methanol (250 µL/L), and each of these four treatments was replicated 3 times.

Seven pairs of *X. laevis* adults were collected from a site where no atrazine was applied in the watershed, and the frogs were induced to spawn. To each microcosm tank, 600 4-day old tadpoles were added; 2 weeks later an additional 200 4-day old tadpoles were added to each tank to achieve an initial population of 800 tadpoles/tank. Water quality parameters (DO, conductivity, pH, air and water temperature were measured weekly. Atrazine concentrations were measured bi-weekly by gas chromatography coupled to mass spectroscopy (GC/MS). Supplementary food (20 g pulverized rabbit pellets) was initially provided once per week but later increased to twice a week.

Extensive mortality was reported in one replicate each of both the 1 and 10 µg/L atrazine treatments, but these two replicates were excluded from the analysis. Metamorphs reaching stage 66 were removed from the microcosms every other day from November 2002 through mid-January 2003 for a total of 150 metamorphs per treatment concentration. It wasn't possible to obtain 50 metamorphs from each replicate, and no more than 75 metamorphs were collected from a single replicate. Metamorphs were euthanized in methyl tricaine sulfonate and snout-vent length and weight were recorded; specimens were then fixed in Bouin's solution and later examined for gross morphology. Histology was conducted on frogs at stage 66 (214 randomly selected frogs at metamorphosis; **Table 38**) and at 10 months of age. Serial sections (6 µm) of the entire gonad were examined for anomalies.

Atrazine concentrations are reported to have remained constant during the first 80 days of the study after which time the microcosms were drained and fresh atrazine solutions were added as frogs grew to maturity. Time-weighted measured concentrations averaged 1.43, 12.1, and 30.8 in the 1, 10, and 25 µg atrazine/L treatments, respectively. Atrazine was detected in one of the control replicates at the method limit of detection (0.1 µg/L); however, no specimens were collected from this replicate. Over the study period, temperature ranged from 10°C to roughly 24°C; pH ranged roughly between 7.4 to 8.8 and dissolved oxygen fluctuated widely and ranged between roughly 2.0 mg/L to 7.5 mg/L. At 24°C and a dissolved oxygen concentration of 2 mg/L, the percent oxygen saturation was roughly 24%.

Table 7. Number of male and females NF Stage 66 frogs collected for histology.

Treatment	Number of Males	Number of Females
Control	23	22
1 µg/L atrazine	30	24
10 µg/L atrazine	17	34
25 µg/L atrazine	31	27

The first frogs completed metamorphosis (NF Stage 66) in the 10th week of the study, and collection of Stage 66 metamorphs was terminated in week 19 when the target number was met. According to the authors, of the 150 NF Stage 66 metamorphs examined from each treatment via gross morphology, few gonadal anomalies were observed (**Table 39**). All of the anomalies were observed in males and consisted only of abnormal segmentation.

Table 39. Number of Stage 66 Frogs examined and the number and percentage of gonadal anomalies based on gross morphology.

Treatment	Number of				Percent Gross Gonadal Anomalies	
	Frogs Collected	Males	Females	Males with Anomalies	Total Frogs	Males
Control	150	68	82	6	4.0	8.8
1 µg atrazine/L	150	72	78	2	1.3	2.8
10 µg atrazine/L	150	59	91	1	0.7	1.7
25 µg atrazine/L	150	70	80	5	3.3	7.1

The only gonadal anomaly observed during histology was the presence of testicular oocytes. The prevalence of testicular oocytes in controls was 57% compared with 57%, 59%, and 39% in the 1, 10, and 25 µg atrazine/L treatments, respectively. There was no statistical difference in the prevalence of testicular oocytes across treatments (p=0.40), and there was no correlation between gross morphological anomalies and the presence of testicular oocytes. There was no statistically significant difference between the incidents of testicular oocytes in only one of both testes (p=0.60), and there was no statistical difference in the number of testicular oocytes per specimen (p>0.27). The majority expressing testicular oocytes had between 2 to 10 oocytes per testis with a maximum of 58 oocytes in the testes of one individual. The prevalence of testicular oocytes as well as the number per individual was less in the 10-month old individuals than in the NF Stage 66 animals. Based on the report figures, roughly 40% of the control males had testicular oocytes compared to 20, 0, and 40% in the 1, 10, and 25 µg of atrazine/L treatments, respectively.

The authors conclude that there is little evidence to suggest that atrazine causes male gonadal anomalies in *X. laevis* at environmentally relevant concentrations and that the occurrence of testicular oocytes is not related to atrazine but rather a natural ontogenetic process.

This is one of the studies previously reviewed (USEPA 2003) as an interim report (Du Preez and Solomon. 2003; MRID 458677-11). The origin of the adult frogs used for spawning purposes is not clearly indicated other than that they came from a muddy pond surrounded by a wall in an area where atrazine was not used. Since macrophytes were permitted to colonize the microcosms, apparently the plants must have been insensitive to atrazine.

It is unclear why 200 additional tadpoles were added after the initial 600 since this would confound efforts to determine time to metamorphosis. Replicates were not sampled equally for histology.

Frogs in early developmental stages (Days 1 – 30) were exposed to relatively cold temperatures (10 – 17°C), and it is unclear what effect this may have had on the prevalence of gonadal anomalies. Animals added 2 weeks after the first batch would have experienced warmer water (by roughly 7°C) during early stages of development compared to the first batch; however, it would not have been possible to distinguish between these animals by Stage 66. Thus, this is considered a confounding effect.

Dissolved oxygen concentrations fluctuated widely during the study, and in the 25 µg atrazine/L treatment, it dropped to approximately 2 µg/L. At 24°C and a dissolved oxygen concentration of 2 mg/L, the percent oxygen saturation would be roughly 24%. Low dissolved oxygen concentrations can influence growth and development; the extent to which the low dissolved oxygen in this study influenced the study animals is uncertain.

The prevalence of testicular oocytes (up to 57% in the controls) is a surprisingly high number that has not been reported for amphibians collected in the wild.

Although length and weight of metamorphs was reportedly recorded, the results were not presented.

The authors report that the water temperatures in this study were lower than those that may have been encountered in the field. At 20 – 25°C, *Xenopus* takes approximately 58 days to reach NF Stage 66, and in its natural habitat, time to metamorphosis ranges between 56 – 63 days. In this study, the first frog completed metamorphosis at 70 days, and frogs were still completing metamorphosis after 133 days.

The prevalence of gonadal anomalies in males (discontinuous gonads) ranged from 1.7 – 7.1% and 8.8% in controls; however, these results were not correlated at all with the much larger prevalence of testicular oocytes (up to 57% in the controls) based on histology. The authors report that 7% of the male *X. laevis* collected in the wild were found to have testicular oocytes;

however, they also note that few studies have conducted in depth histological analysis and may have underestimated the actual prevalence of testicular oocytes.

Hecker, M., J. P. Giesy, P. D. Jones, A. M. Jooste, J. A. Carr, K. R. Solomon, E. E. Smith, G. Van Der Kraak, R. J. Kendall and L. Du Preez. 2004. Plasma Sex Steroid Concentrations and Gonadal Aromatase Activities in African Clawed Frogs (*Xenopus laevis*) from South Africa. *Environmental Toxicology and Chemistry*. 23(8): 1996 – 2007.

Description of Study and Results

In this study, five corn-growing region (CGR) ponds were selected based on the presence of *X. laevis* and the proximity to corn production. Additionally, three non-corn growing region (NCGR) ponds were selected based on the absence of corn production. CGR sites were situated in Viljoenskroon (Free State Province, South Africa), while NCGR were near Potchefstroom (North West Province, South Africa). Sites were selected to give a range of concentrations of atrazine and other pesticides used in corn production. Compounds of major interest were atrazine and its degradation products deethylatrazine (DEA), deisopyrpylatrazine (DIA), diaminochloroatrazine (DACT), and tertbuthylazine; simazine and acetochlor were also measured in this study. The limit of detection (LOD) for simazine was 0.5 µg/L, and the LOD for other triazines was 0.1 µg/L.

NCGR pond surface areas ranged from 2,000 – 20,500 m^2 and ranged in maximum depth from 38.5 to 261 cm. The pH ranged from 5.1 to 8.8 across the ponds. One of the three NCGR ponds was described as "drying up frequently," likely owing to its shallow depth (38.5 cm) and small surface area (2,000 m^2). The three ponds had catchment areas that ranged between 170 to 480 hectares, and two of the three ponds were spring-fed.

CGR pond surface areas ranged between 2,400 to 68,000 m^2. No information was provided on the maximum depth of these ponds, and no information was provided on whether any of the CGR ponds were spring-fed. The pH ranged from 7.2 to 10.8 across the CRG ponds.

Adult African clawed frogs (*X. laevis*) were collected after the rainy season in April and May 2002. A total of 20 male and 20 females were collected from each of the NCGR ponds. Frogs were less abundant in the CGR ponds (reportedly due to predatory catfish), and therefore only 10 males and 10 females were collected from each of these ponds except for one pond where

only 3 males and 8 females could be collected. Frogs were collected in traps baited with liver and marrow bones. Traps were checked two days later, and frogs were then transferred to the lab.

Individual frogs were acclimated [in lab] in 2-L plastic containers in lab water for 48 hours. After blood was collected via cardiac puncture, it was then centrifuged and the plasma stored at -80^{o}C. Gonads were removed, measured, and weighed. One gonad was then flash frozen in liquid nitrogen, while the other gonad was fixed in 10% neutral formalin for eventual histology. The gonadal somatic index (GSI) was calculated as:

GSI= (gonad weight÷body weight) X 100

Plasma estradiol and testosterone levels were measured using competitive ELISA. CYP19 aromatase activity in the gonadal tissue was measured using the tritiated water release assay described by Lephart and Simpson (1991).

Recoveries of estradiol in males and females were 96±13% (n=4) and 87±13% (n=7), respectively; recoveries of testosterone in males and females were 90±28% (n=10) and 113±19% (n=19), respectively.

Mean-measured atrazine concentrations in NCGR pond water ranged from 0.13 to 0.47 µg/L; the peak measured atrazine concentration was 0.57 µg/L, and the peak degradate (DACT) level was 3.91 µg/L. The highest concentration of tertbutylazine was 0.59 µg/L. Mean-measured atrazine concentrations in CGR ponds ranged from 0.31 to 3.82 µg/L. The peak measured atrazine concentration was 4.14 and the peak degradate (DACT) concentration was 5.45 µg/L. The highest concentration of tertbutylazine was 3.66 µg/L.

There was no significant difference ($p>0.05$) in GSI values for either males or females collected from CGR and NCGR although the authors note that the ratio appeared to be higher for animals collected from CGR ponds. There was less variability in GSI for animals collected from NCGR. GSI for males and log GSI for females were significantly and positively correlated with the log conductivity. The GSI for males was significantly and negatively correlated with the log visual depth. Female GSI was significant ($p=0.026$) and negatively correlated with mean molybdenum concentrations in the sediment and was significant ($p=0.005$) and positively correlated with mean strontium ion concentrations.

Median plasma testosterone concentrations in females were significantly (p=0.018) greater at NCGR ponds; however, there was no difference between males collected from the two regions. Plasma testosterone concentrations were negatively correlated with the log atrazine concentration (r= ¯0.934; p=0.001) and tertbutylazine (r= ¯0.736; p=0.028). Log testosterone concentration in females was also significant and positively correlated (r=0.845; p=0.008) with mean vanadium concentration. Log plasma testosterone concentration in males was significant and negatively correlated with the log of the DACT concentration (r= ¯0.831; p=0.011).

Median plasma estradiol of females from CGR was significantly less than from NCGR (p=0.002); however, there was no difference for males. There was a significant positive correlation between log testosterone and log estradiol concentrations in both males (r= 0.757; p<0.001) and females (r= 0.868; p<0.001).

The ratio of estradiol to testosterone in both males and females was not significantly different between the two regions regardless of whether the comparisons were made between medians or means. The only significant correlation between the estradiol to testosterone ratio for males was with DIA (r= ¯0.830; p=0.011). Significant negative correlations were observed between the log estradiol-testosterone ratio for males and both log temperature (r=0.810; p=0.015) and dissolved oxygen (r=0.717; p=0.045). The estradiol-testosterone ratio for both sexes was positively correlated with the concentration of aluminum in the water. Both sexes also exhibited positive correlations between log plasma estradiol concentration and the log estradiol-testosterone ratio (males r=0.804, p<0.001; females r=0.580, p<0.001).

No significant difference was observed in the median aromatase activity in ovaries between the two collection regions. Aromatase concentrations were below the limit of detection for testes in most sites. Log ovarian aromatase activity was significant and negatively correlated with nitrate (r= ¯0.787; p=0.02), nitrite (r= ¯0.776; p= 0.024), silicon (r= ¯0.812; p=0.014), titanium (r= ¯0.763; p=0.037) and vanadium (r= ¯0.721; p=0.044). Aromatase activity was not correlated with any of the pesticides measured. Aromatase activity was not correlated with either plasma testosterone or estradiol concentrations in either males or females.

Differences in female plasma testosterone and estradiol concentrations between NCGR and CGR and significant correlations between female plasma sex steroid levels and atrazine and

its degradates are noted. Additionally, the significant negative correlation between male plasma testosterone levels and DACT concentration is noted. However, the authors state that few studies have reported effects of atrazine on plasma estradiol concentrations, but that atrazine exposure has been associated with decreased, not increased, testosterone levels.

The authors conclude that the study results do not support the hypothesis that atrazine causes an increase in the production of estradiol by inducing aromatase activity; however, they acknowledge that the statistical power of the study to discriminate the potential effects of atrazine on aromatase activity is low.

The authors speculate that the significant positive correlation between estradiol and testosterone in both males and females indicates that both hormones are equally affected and this effect may be attributed to increased metabolism. They also speculate that atrazine may produce effects on the endocrine system at the level of the central nervous system.

Analysis

This is one of the studies previously reviewed (USEPA 2003) as an interim report (Smith *et al*. 2003a; MRID 458677-10). Fewer frogs were obtained from all of the CGR ponds than from the NCGR ponds reportedly due to predatory catfish that had moved into the ponds as a result of flooding from high rain events. This suggests that the ponds do not represent discrete sample units but are at times contiguous with a larger river system.

Atrazine was detected in NCGR ponds albeit at levels below those at the CGR ponds; however, some of the CGR ponds had measured atrazine concentrations that were roughly equivalent to the NCGR ponds. Additionally, the second highest measured concentration of the atrazine degradate DACT occurred in a NCGR pond. These data suggest that although NCGR ponds were not in close proximity to atrazine, they nevertheless contained atrazine and its degradates at levels that overlap with those of CGR ponds.

The lower variability associated with GSI from NCGR may be due to the higher sample size (N).

The authors note several correlations between GSI and abiotic factors in ponds, and plasma testosterone concentrations were correlated with vanadium concentrations. The relationships to molybdenum, strontium, and vanadium suggest that there were sufficiently high concentrations of these metals to conduct a correlation analysis. Although actual concentrations of various metals are not reported, the presence of measurable concentrations of titanium, chromium, molybdenum, vanadium, and strontium in the water column and in sediment could suggest that there were other sources of contamination besides herbicides in corn production and that these metals could potentially impact gonadal development and steroidogenesis. This was an issue that was identified in South African field studies of atrazine previously reviewed by the Agency (Smith *et al*. 2003a; Du Preez *et al*. 2005).

The authors note that the lack of any statistical difference for either plasma testosterone or estradiol in males collected from CGR and the NCGR may be caused by the inability of the study to discriminate effects due to low sample size.

Although the authors state that the ratio in males was significant and positively correlated with aluminum (r=0.845; p=0.008), it was significant for sediment chromium ion concentration as well (r=0.845; p=0.008).

Although the authors state that testicular aromatase activity was below the limit of detection for many of the animals sampled, they report significant negative correlations with nitrate, nitrite, silicon, and titanium. The authors do not describe how nondetections were accounted for in these analyses.

The authors discuss how only trace amounts of atrazine and other triazines were detected in NCGR ponds; however, the data suggest that triazine levels were comparable at some locations in both regions. As stated previously, the second highest concentration of the atrazine degradate DACT was reported for a NCGR pond.

The authors note that previous field studies have suggested that atrazine exposure may affect the prevalence of gonadal abnormalities. In this study, no significant correlation was observed in this study between the concentrations of atrazine and degree of response. Although this study reports that gonad samples were collected for histology, the study does not provide any information on the incidence of gonadal abnormalities; therefore, the degree of response alluded

to by the authors must be in reference to GSI, plasma hormone, and gonadal aromatase activity measured in the current study. However, the authors repeatedly state in this study that the statistical power to discriminate effects was low. The fact that atrazine degradates were detected at relatively high concentrations in NCGR sites implies that triazines were present at potentially similar concentrations as in CGR sites, thus confounding the ability of the study to differentiate between NCGR and CGR sites. Finally, the significant correlations between many of the measurement endpoints and heavy metals suggest that there were additional confounding effects. While chromium, titanium, vanadium, and strontium are all naturally occurring elements, their presence at concentrations sufficient to establish correlations suggests that the sampling sites may have been affected by heavy metal contamination beyond background.

GSI was significant and positively correlated with atrazine concentrations at CGR sites and the study authors hypothesize that this may be a result of differences in food availability due to increased nutrient loading. The authors speculate that the positive correlation between GSI and conductivity support this hypothesis. However, neither male nor female GSI was correlated with either nitrate or nitrite, both of which are typical indices of nutrient loading to aquatic systems.

The authors speculate on the reasons for differences in plasma sex steroid concentrations measured in the current studies to those reported in the open literature and suggest that laboratory-reared animals, non-wild stock, and normal mating seasons can influence sex steroid levels. However, it is also possible that the collection method, *i.e*., trapping, may have placed animals in sufficiently close proximity to one another for up to 2 days to initiate mating behavior and in turn stimulate plasma sex steroid levels. Although the authors state that female plasma estradiol concentrations for frogs collected in NCGR were indicative of animals at the peak of their reproductive activity, they also state that the frogs were collected in the late or post-spawning period and that the variability observed in both testosterone and estradiol concentrations was likely a result of collecting frogs at the end of their breeding season.

Although the authors conclude that the study provides evidence that female plasma estradiol and testosterone concentrations are less in CGR compared to NCGR where concentrations of pesticides, including atrazine are present, they state that the ranges overlapped and that the differences were not likely to result in biologically relevant effects at the population level. To support this conclusion, they note that gonad growth was not affected and that robust populations of frogs were present at both locations. The results of this study suggest, however,

that the two regions were not sufficiently distinct in terms of potential triazine levels and were in some cases remarkably similar. Additionally, the study does not provide a strong case for similar robust frog populations in both regions given the difficulty that the researchers had in obtaining sufficient numbers of frogs in the CGR.

The authors state that there was no evidence to suggest that differences in plasma sex hormone levels were caused by an induction of aromatase. To support this conclusion, they note that a number of other factors (*e.g.*, "selected ions") showed significant correlations with both atrazine concentrations and the measurement endpoints. Because of this correlation, the authors contend that it is "impossible to determine the extent and mode of action by which atrazine may affect the endocrine system of *X. laevis*." While the correlations noted in this study do not establish causality; they suggest that the ability of the study to examine the potential effects of atrazine on plasma sex hormone levels and gonad aromatase activity may have been confounded by many factors. Triazine levels between the two sampling regions may not have been sufficiently different, and the level of heavy metals in the sampling regions may have further confounded the study.

This study underscores the difficulties associated with conducting field studies and how relationships should be more thoroughly vetted and established under controlled laboratory conditions before attempting to test hypotheses under field conditions. Collection methods may have increased biological variability within and between regions; however, abiotic factors and actual pesticide concentrations across the sampling areas appear to have further confounded the studies ability to discriminate effects should they exist.

Du Preez, L. H., P. J. Jansen van Rensburg, A. M. Jooste, J. A. Carr, J. P. Giesy, T. S. Gross, R. J. Kendall, E. E. Smith, G. Van Der Kraak and K. R. Solomon. 2005. Seasonal Exposures to Triazine and other Pesticides in Surface Waters in the Western Highveld Corn-production Region in South Africa. Environmental Pollution 135 (2): 131 – 141.

Description of Study and Results

The objective of this study was to determine concentrations of atrazine, terbuthylazine, their degradation products, and other pesticides in surface waters in amphibian habitats in the study areas during the 2001 – 2002 growing season.

Sites located in corn-growing areas (CGA) were located near the Viljoenskroon area while non-corn growing area (NCGA) reference sites were located 50 km to the north in the

Potchestroom area. Two of the three NCGA sites were fed by intermittent springs, while the third was fed by rainfall and surface water runoff. NCGA ponds ranged in surface areas from 0.2 to 2.05 ha. Two of the ponds were located on cattle farms whereas the third pond was located in a security zone outside a munitions factory. CGA ponds ranged in surface area from 0.22 to 6.87 ha and were located in grasslands mixed with cornfields.

At each site, four 1-liter water samples were collected and combined. From the combined sample, two 1-liter samples were collected. Water samples were kept cool during transport back to lab where they were stored at 4^{o}C until analysis. Water samples were collected weekly from Nov 5, 2001 to Feb 18, 2002. Afterwards, samples were collected at 2-week intervals until June 10, 2002. Sediment samples were also collected. At each sampling period, water temperature, conductivity, dissolved oxygen, and pH readings were recorded.

Atrazine was measured using two methods, one with solid-phase extraction (C-18) and the second with liquid-liquid extraction. All analyses were performed using an Agilent model 6890 Series II-Plus gas chromatograph interfaced to a 5973 mass selective detector operated in the selected ion monitoring mode. The level of detection (LOD) for atrazine, its degradates and terbuthylazine was 0.1 µg/L. The LODs for simazine, acetochlor, S-metolachlor, cypermethrin, monocrotophos, and terbuphos were 0.5 µg/L.

According to the authors, only trace amounts of atrazine (average 0.4 µg/L) were detected in the NCGA ponds although they report an "unexpected spike" of atrazine in one of the ponds. Concentrations of atrazine at CGA increased after corn was planted and herbicides were applied with values ranging from 4 to 10 µg/L. Terbuthylazine was detected at concentrations up to 4.1 µg/L in the CGA sites, while simazine was detected at up to 3.2 µg/L in the CGA sites.

Concentrations of triazine degradation products deisopropylatrazine (DIA) and deethylatrazine (DEA) were detected at all sites one or more times during the monitoring period. DEA was measured up to 2.2 µg/L, while DIA was ≤1 µg/L. DACT ranged from <LOD to approximately 8 µg/L.

Trace element analysis indicated differences between CGA and NCGA sites that were attributed to differences in the geology of the sites. The authors noted that some elements detected in the water were at concentrations that exceeded the South African Water Quality

Guidelines for Aquatic Ecosystems. They also noted that aluminum, iron, and silica were present in relatively large concentrations in one of the NCGA ponds.

Analysis

This is one of the studies previously reviewed (USEPA 2003) as an interim report (Smith *et al*. 2003b; MRID 458677-09). Although the study authors claim that atrazine was detected at trace levels in NCGA ponds, measured concentrations averaged 0.4 µg/L, and the authors report a spike in atrazine concentrations in one of the reference ponds. The "spike" in atrazine concentrations in the reference pond appeared to persist for roughly 30 days at concentrations close to 1 µg/L (**Figure 13** depicts Figure 6 of the report showing atrazine and terbuthylazine concentrations).

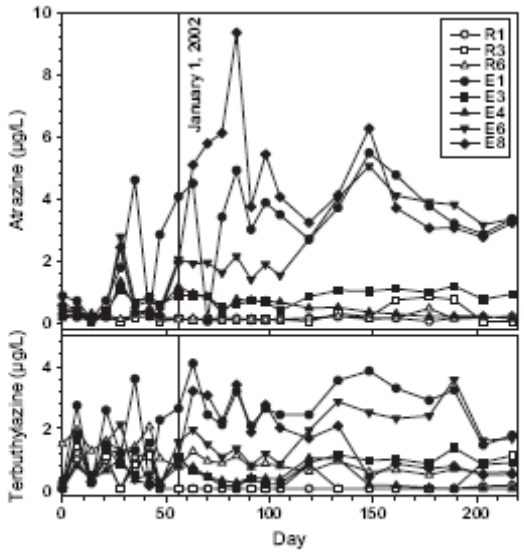


Fig. 6. Atrazine and terbuthylazine concentrations measured at the NCGA and CGA sites. Each point represents a single analysis.

Figure 13. Measured atrazine and terbuthylazine concentrations in surface water collected from non corn-growing areas (NCGA) and corn-growing areas (CGA) in South Africa. Figure represents Figure 6 depicted in Du Preez *et al*. 2005.

Although the study authors report that terbuthylazine was detected at concentrations up to 4.1 µg/L in CGA sites and at lower concentrations in NCGA sites, they do not describe the actual measured concentrations at the NGCA sites. From **Figure 13,** it appears that measured concentrations of terbuthylazine peaked at roughly 2 µg/L several times during the study period.

Based on measured concentrations of the atrazine degradates DIA, DEA, and DACT (**Figure 14** depicts Figure 7 of the study), the highest measured concentrations of DIA and DEA appear to be associated with the NCGA ponds. Peak concentrations of DACT were roughly 7 µg/L at one of the NCGA ponds and from roughly 90 days onward in the study peaked at concentrations above those measured in CGA ponds.

Although the authors reported that there were differences in the trace elements detected between NCGA and CGA ponds, they do not provide much analysis other than to say that concentrations of aluminum, iron, and silica were relatively high in NCGA ponds. **Table 40** and **41** depict Tables 3 and 4 from the paper and report trace element concentrations in both surface water (**Table 40**) and sediment (**Table 41**). Aluminum concentrations were as high as 33.8 mg/L, chromium was as high as 0.016 mg/L, titanium was as high as 0.6 mg/L; and iron was as high as 21.3 mg/L in surface water collected from reference ponds in the NCGA. In sediment, chromium was as high as 100.5 mg/kg in NCGA ponds.

Additionally, in reference NCGA ponds, pH appears to fluctuate widely and drops to 5 on at least two separate occasions. In CGA, pH values were high relative to the NCGA ponds; pH values reached as high as 10 in CGA ponds. Low pH ponds in combination with high aluminum levels could prove detrimental to amphibian survival.

CGA ponds were distinctly different from NCGA ponds in that the former had high conductivity. In general, NCGA ponds were an order of magnitude less conductive than CGA ponds, and in some cases conductivity differed by as much as 64X.

Although this study does not provide information on the effects of atrazine alone on amphibian gonadal development, it does provide information on environmental conditions in a study area used to collect amphibians. These data were reported in an interim study by Smith *et al*. 2003b[4] (MRID 458677-09) that was reviewed for the 2003 SAP. The data are also relevant to a recently submitted study by Du Preez *et al.* 2005[5] discussing the population structure of African

4 Smith, EE, Du Preez, L, and Solomon, K. 2003b. 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. Ecorisk, Inc. Final Report SA-01C, Submitted to Syngenta Crop Protection, Inc., Dated February 18, 2003, 104 pp.

5 Du Preez, L.H., Solomon, K.R., Carr, J.A., Giesy, J.P. Gross, T.S., Kendall, R.J. Smith, E.E., Van der Kraak, G. & Weldon, C. (2005). Population structure characterization of the African Clawed Frog (*Xenopus laevis*) in maize-growing versus non-maize-growing areas in South Africa. *African Journal of Herpetology* 54(1): 61-68.

clawed frogs (*Xenopus laevis*) in corn-growing regions and a microcosm study by Jooste *et al.* 2005[6]; the latter study was also reviewed as an interim report by Du Preez and Solomon (MRID 458677-11[7]) for the 2003 SAP.

As discussed in previous reviews of interim reports, the environmental conditions in some of the ponds selected as reference sites are such that amphibian growth and survival may have been impaired from the presence of heavy metals. Additionally, atrazine concentrations and concentrations of atrazine degradates such as DACT in reference ponds in NCGA were at some sampling times equivalent to and/or exceeded values in CGA areas. As such, it would be difficult to attribute any differences in amphibian development to any particular factor due to the number of potential confounding effects. Because of the relatively high levels of metals and triazine compounds in the reference ponds, any study that relied on animals collected from these sites would be considered compromised.

6 Jooste A. M., L. H. Du Preez, J. A. Carr, J. P. Giesy, T. S. Gross, R. J. Kendall, E. E. Smith, G. J. Van Der Kraak, and K. R. Solomon. 2005. Gonadal development of *Xenopus laevis* larvae exposed through larval development to atrazine in outdoor microcosms. *Environmental Science and Technology* 39:5255-5261.

7 Du Preez, L, and Solomon, K. 2003. Exposure Xenapus larvae to different concentrations of atrazine in semi-natural microcosms. Ecorisk, Inc. Final Report SA-01D, Submitted to Syngenta Crop Protection, Inc., Dated February 16, 2003, 44 p.

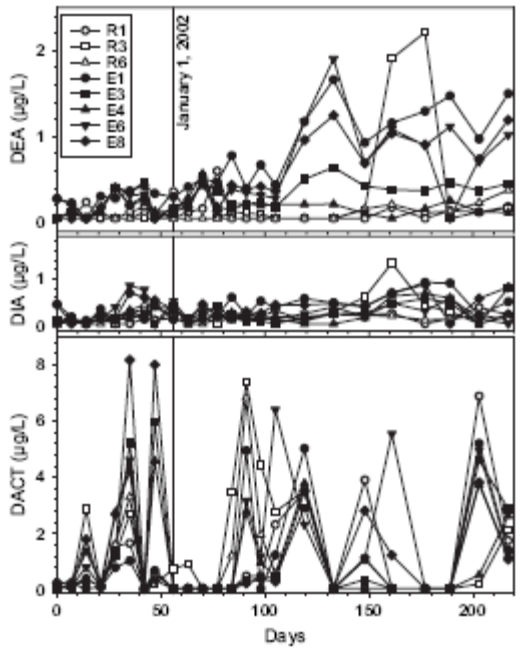


Fig. 7. Concentrations of the triazine degradation products, DEA, DIA, and DACT measured at the NCGA and CGA sites. Each point represents a single analysis.

Figure 14. Measured concentrations of DEA (top), DIA (middle), and DACT (bottom) in non corn-growing area (NCGA) and corn-growing area (CGA) ponds. Figure depicts Figure 7 from Du Preez *et al.* 2005.

Table 40. Trace element analysis in surface water collected from non-corn-growing area (NCGA) and corn-growing area (CGA) ponds in South Africa. Table represents Table 3 from Du Preez *et al.* 2005.

Table 3
Mean concentrations of elements and ions (mg/L) in unfiltered water at reference and exposed sites

Elements and ions[a]	R1	R3	R6	E1	E3	E4	E6	E8	Guideline[b]
Aluminum as Al	6.0	2.0	33.8	1.3	0.2	0.1	0.02	0.03	0.1
Antimony as Sb	ND	ND	ND	ND	ND	ND	ND	ND	NA
Arsenic as As	ND	ND	ND	ND	ND	ND	ND	ND	0.005
Barium as Ba	0.01	0.1	0.2	0.1	0.02	0.1	0.1	0.1	NA
Beryllium as Be	ND	ND	ND	ND	ND	ND	ND	ND	NA
Bismuth as Bi	ND	ND	ND	ND	ND	ND	ND	ND	NA
Boron as B	ND	ND	ND	ND	ND	ND	ND	ND	NA
Cadmium as Cd	ND	ND	ND	ND	ND	ND	ND	ND	0.000017
Calcium as Ca	2.3	4.0	4.5	25.4	31.6	30.8	33.2	33.2	NAp
Chromium as Cr	0.012	0.010	0.016	0.028	0.014	0.014	0.004	0.004	0.001
Cobalt as Co	ND	ND	ND	ND	ND	ND	ND	ND	NA
Copper as Cu	ND	ND	ND	ND	ND	ND	ND	ND	0.002
Iron as Fe	4.5	2.1	21.3	0.8	0.2	0.7	ND	ND	0.3
Lead as Pb	0.018	0.016	0.034	ND	ND	ND	ND	ND	0.001
Lithium as Li	ND	ND	ND	ND	ND	ND	ND	ND	NA
Magnesium as Mg	3.1	3.8	3.2	28.6	27.4	27.0	28.4	28.4	NAp
Manganese as Mn	ND	ND	0.1	ND	ND	0.2	ND	ND	NA
Mercury as Hg	ND	ND	ND	ND	ND	ND	ND	ND	NA
Molybdenum as Mo	ND	ND	ND	ND	ND	ND	ND	ND	0.073
Nickel as Ni	ND	ND	ND	ND	ND	ND	ND	ND	0.025
Nitrate as NO_3	0.4	0.4	0.6	0.6	0.7	0.7	0.3	0.4	NA
Nitrite as NO_2	0.3	0.4	0.4	0.5	0.5	0.5	0.4	0.4	0.06
Phosphorus as P	0.01	0.1	0.1	0.1	0.7	0.9	0.4	0.3	NAp
Potassium as K	1.2	2.5	7.4	12.2	19.0	19.2	11.4	11.4	NAp
Selenium as Se	ND	ND	ND	ND	ND	ND	ND	ND	0.001
Silica as Si	8.7	4.7	49.2	5.5	5.8	4.8	4.3	4.3	NAp
Silver as Ag	ND	ND	ND	ND	ND	ND	ND	ND	0.0001
Sodium as Na	2.4	2.8	1.3	63.6	106.4	115.8	55.2	55.2	NAp
Strontium as Sr	ND	0.02	0.03	0.1	0.2	0.2	0.2	0.2	NA
Sulphate as SO_4	3.0	3.2	6.2	23.6	29.5	25.7	26.7	26.7	NAp
Sulphur as S	1.6	2.0	2.1	9.1	10.7	10.4	9.6	9.6	NA
Thallium as Tl	ND	ND	ND	ND	ND	ND	ND	ND	0.0008
Tin as Sb	0.2	ND	0.2	ND	0.2	0.6	0.6	0.6	NA
Titanium as Ti	0.1	0.03	0.6	0.1	0.01	ND	ND	ND	NA
Vanadium as V	0.01	ND	0.01	ND	ND	0.002	ND	ND	NA
Zinc as Zn	ND	ND	ND	ND	ND	ND	ND	ND	0.03
Zirconium as Zr	ND	ND	ND	ND	ND	ND	ND	ND	NA
Total hardness as $CaCO_3$	18.2	25.0	24.6	182.0	191.4	188.2	200.4	200.4	NAp

[a] Mean of five monthly samples except for nitrate and nitrite which are based on seven samples.
[b] From (CWQG, 1999). NA = not available. NAp = not applicable.

Table 41. Trace element analysis in benthic sediments collected from non-corn-growing area (NCGA) and corn-growing area (CGA) ponds in South Africa. Table represents Table 4 from Du Preez *et al.* 2005.

Table 4
Concentrations of elements in sediments at NCGA and CGA sites

Element[a]	Units	R1	R3	R6	E1	E3	E4	E6	E8
Arsenic as As	mg/kg	ND	ND	ND	ND	ND	ND	ND	ND
Cadmium as Cd	mg/kg	ND	ND	ND	ND	ND	ND	ND	ND
Chromium as Cr	mg/kg	7.01	ND	100.50	7.51	20.00	22.00	19.00	57.50
Copper as Cu	g/100 g	ND	0.01	0.01	0.06	0.01	0.17	0.03	0.02
Iron as Fe	g/100 g	2.89	3.16	1.05	7.06	5.06	2.61	5.05	9.38
Lead as Pb	mg/kg	0.31	0.18	ND	ND	0.02	ND	ND	0.10
Mercury as Hg	mg/kg	ND	ND	ND	ND	ND	ND	ND	ND
Molybdenum as Mo	mg/kg	0.03	0.04	0.02	ND	ND	ND	0.01	0.01
Nickel as Ni	mg/kg	ND	ND	ND	ND	ND	ND	ND	ND
Selenium as Se	mg/kg	ND	ND	ND	ND	ND	ND	ND	ND
Silver as Ag	mg/kg	ND	ND	ND	ND	ND	ND	ND	ND
Thallium as Tl	mg/kg	ND	ND	ND	ND	ND	ND	0.01	ND
Zinc as Zn	g/100 g	ND	ND	ND	0.25	0.09	0.03	ND	ND

[a] Based on the mean of five monthly samples.

Smith, E. E., T. A. Anderson, C. Crabtree, J. A. Carr, L. H. Du Preez, A. B. Gentles, J. P. Giesy, T. S. Gross, L. Holden, R. J. Kendall, K. R. Solomon, and G. Van Der Kraak. 2007. Reproductive Characteristics in American Bullfrogs (*Rana catesbeiana*) Collected from Agricultural and Non-Agricultural Areas in Southern Iowa. Submitted to *Environmental Toxicology* (MRID 472045-01)

Description and Results of Study

The objective of this study was to examine aspects of reproductive status in native North American anuran species, *i.e.,* the bullfrog (*Rana catesbeiana*) collected from agricultural areas (AA) and nonagricultural areas (NAA) in Iowa. Reproductive status was examined through gonadal morphology, plasma testosterone, and plasma estradiol concentrations in relation to atrazine and its metabolite (desethyl atrazine [DEA] and deisopropylatrazine [DIA]) concentrations.

Seven ponds were selected in agricultural areas based on their proximity to corn/soybean production, previous/planned use of atrazine, and the presence of bullfrogs. Three sites were selected in nonagricultural areas based on their relative isolation from corn/soybean production and the presence of bullfrogs. NAA and AA ponds ranged in size from 0.49 - 1.62 and 0.14 – 2.94 ha, respectively. Watershed areas for NAA and AA ponds ranged from 5.8 – 84 ha and from 2.2 – 31.3 ha, respectively. NAA watersheds consisted primarily of turf, residential/turf or hardwood forest. The authors stated that there were no reported herbicide applications for the NAA sites. Each site was sampled four times: May 27 – June 1, June 22 – 27, August 3 – 8, and September 11 – 15. Duplicate 250-mL water samples were collected from four sampling stations around each of the study ponds; each of the individual water samples were then pooled and shipped on ice to Texas Tech University for analysis using high pressure liquid chromatography equipped with a UV detector. No other pesticides were analyzed from the AA. The analytical limit of quantification (LOQ) was 0.22 µg/L, 0.50 µg/L, and 0.40 µg/L for the parent atrazine, DIA, and DEA, respectively. Nondetections were recorded as ½LOQ; concentrations reported in the study represent total atrazine residues, *i.e*., atrazine+DEA+DIA.

Frogs were collected at night from each study site, at four time periods, for a maximum of 1 hour per sampling period or until 17 frogs were collected. Frogs were maintained in buckets containing pond water and returned to the lab.

A total of 458 bullfrogs were collected and out of this total only 13 had not completed metamorphosis. Of these, there were 235 males, 209 females, and 1 unidentified gender. Animals were then classified by weight as either juveniles (≤60 g) or adults (>60 g).

Animals were anesthetized using tricaine methanesulfonate and measured (body weight and snout-vent length); blood samples were then drawn by cardiac puncture; afterward, digital photographs were taken of the gonads. The right gonad was then removed, weighed, and flash frozen in liquid nitrogen for hormone analysis while the left gonad prepared for histology.

Plasma testosterone was measured using a Coat-a-Count® radioimmunoassay Kit (Diagnostic Products Corporation), while plasma estradiol was measured by radioimmunoassay (Polymedco MAIA® kit).

Atrazine and DEA concentrations were below the LOQ in all of the NAA ponds across the entire sampling period, while total residues in AA ponds ranged between <LOQ to 41.2 µg/L. **Table 42** (depicting Table 1 of the report) does not state whether the atrazine concentrations for each pond and month represent average or peak concentrations.

According to **Table 42** (depicts Table 1 of report) of the study, temperature across all of the ponds ranged between 23 – 25°C; pH in the NAA and AA ponds ranged between 8.1 – 8.7 and 6.9 – 8.9, respectively. Dissolved oxygen is reported to have ranged between 62.4 – 71.2 mg/L and 26.8 – 73.3 mg/L at the NAA and AA sites, respectively.

According to the study, there was a strong correlation between body weight and snout-vent length, and the slope of the regression analysis was 3. Condition factors, calculated as ratio of SVL to body weight, were not significantly different for either males or females collected at NAA and AA ponds. There was a significant difference (p=0.049) in median condition index for males collected at NAA sites compared to males from AA sites over the four sampling periods; males from NAA sites had smaller median condition index. For both males and females, median condition indices were smaller for frogs collected earlier in the sampling year compared to those frogs collected later in the year.

Body weights ranged from 3.2 – 302 g and 3.8 – 462 g in males and female bullfrogs, respectively. There was no difference in body weight by sex between the NAA and AA sites

when taken as a whole; however, for males collected in June, there was a significant difference ($p=0.006$) in median body weights between areas. Median body weights for female bullfrogs collected from NAA sites in July were significantly larger ($p=0.036$) than female bullfrogs collected from AA sites.

There was no significant difference in GSI for either males or females between sampling sites; however, there were significant differences across season with larger GSI values in both sampling areas for frogs collected in May compared to the other sampling periods.

There were no significant differences in sex ratio between AA and NAA sites. Of the 193 males from AA sites examined for gross gonadal deformities, four (1.6%) exhibited abnormalities including discontinuous or segmented testes. Although bilateral size differences between testes were noted, these differences were not considered as abnormalities. None of the 42 males collected from NAA sites exhibited gonadal abnormalities based on gross morphology.

According to the report, a subsample of 80 males collected form AA and NAA sites in August and September were evaluated for spermatogenesis. There were no differences in fractional volume of spermatogonia, spermatocytes, or spermatozoa between sites or between seasons. Based on histological analyses, only one AA male, sampled in September, was found to have testicular oocytes; the authors conclude that this represents an incidence of 1.3% across the two sampling areas.

There were no statistical differences in plasma testosterone or estradiol for males collected from AA and NAA sites. Male plasma testosterone concentrations averaged $4.6 \times 10^3 \pm 9.5 \times 10^2$ pg/mL and 3.4×10^3 pg/mL $\pm 3.2 \times 10^2$ at NAA and AA sites, respectively. Male plasma estradiol concentrations averaged 6.4 ± 1.3 pg/mL and 7.3 ± 7 pg/mL at NAA and AA sites, respectively.

The authors conclude that their evaluation of reproductive status of male and female bullfrogs inhabiting ponds under field conditions in NAA and AA areas in southern Iowa indicated limited or no differences in spermatogenesis, gonad weight, body weight, or plasma sex steroid levels in male bullfrogs form these two regions. Based on their analysis, the authors conclude that exposure to agricultural environmental conditions in southern Iowa did not result in any observable effect on growth and development of the bullfrogs.

Analysis

This is one of the studies previously reviewed (USEPA 2003) as an interim report (Crabtree *et al*. 2003; MRID 458677-05). Although the authors stated that no herbicides were reportedly used in the NAA sites, at least two of the sites consisted of turf and residential settings in which herbicides could have been used.

The authors state that no pesticides other than atrazine and its two degradates were measured in the AA sites. The report does not state whether pesticides were measured at the NAA sites. Exposure to other pesticides including other triazine herbicides is a major uncertainty in this study.

Although limited pesticide residue analyses were conducted at the time of sampling, it is unlike that the data represent peak residue levels in the ponds. Since the majority of bullfrogs collected were post-metamorphosis, chemical residue data provide no information on what pesticide concentrations larvae may have been exposed to.

The report stated that blood could not be collected from all post-metamorphs due to their size; however, it did not state the actual number of frogs from which adequate blood could be collected.

According to **Table 43** of the study, temperature across all of the ponds ranged between 23 – 25^{o}C; pH in the NAA and AA ponds ranged between 8.1 – 8.7 and 6.9 – 8.9, respectively. Dissolved oxygen is reported to have ranged between 62.4 – 71.2 mg/L and 26.8 – 73.3 mg/L at the NAA and AA sites, respectively.

According to the report, dissolved oxygen concentrations across both types of study areas ranged between 26.8 – 73.3 mg/L. This value is likely in error since such dissolved oxygen concentrations exceed the saturation limit for water. Even under supersaturated conditions, dissolved oxygen does not typically reach such levels. If the dissolved oxygen concentrations are off by a factor of 10, then some of the ponds experienced low mean dissolved oxygen (2.6 mg/L) concentrations that could have made it difficult to sustain aquatic life.

Table 42 Concentration of atrazine, deisopropyl, and desethyl atrazine in surface water and physical properties from study sites in non-agricultural areas (NAA) and agricultural areas (AA) in souhern Iowa. Table represents Table 1 of Smith *et al.* 2007.

Table 1. Concentrations of atrazine, deisopropyl, and desethyl atrazine in surface waters and physical parameters from study sites in Non-Agricultural Areas (NAA) and Agricultural Areas (AA) of Southern Iowa[a].

Site		Site area	Watershed area		May	June	Aug	Sep	May	June	Aug	Sep	Mean Physical Parameters			
		(ha)	(ha)		Atrazine (µg/L)[b]				Desethyl atrazine (µg/L)[c]				T	pH	DO	Cond
													°C		(mg/L)	(µS/cm)
NAA	NAA-1	0.49	9.9		<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	25	8.7	62.4	231.5
	NAA-2	1.05	5.8		<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	25	8.1	64.9	130.0
	NAA-3	1.62	84.0		<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	24	8.5	71.2	185.3
AA	AA-1	0.88	31.3		<LOQ	<LOQ	<LOQ	2.00	<LOQ	<LOQ	<LOQ	<LOQ	23	6.9	30.6	214.8
	AA-2	0.20	2.2		<LOQ	2.90	1.60	2.00	<LOQ	<LOQ	<LOQ	<LOQ	23	7.7	61.1	248.3
	AA-3	2.94	31.3		1.30	<LOQ	2.40	2.90	2.50	<LOQ	<LOQ	<LOQ	24	8.6	73.3	160.3
	AA-4	1.19	14.1		2.50	2.90	11.90	8.60	0.70	1.10	1.80	1.00	25	8.2	26.8	205.5
	AA-5	0.14	3.8		6.40	<LOQ	<LOQ	<LOQ	3.40	4.60	3.60	0.40	23	8.9	56.8	235.8
	AA-6	0.37	11.6		9.80	<LOQ	11.60	10.60	<LOQ	4.30	0.80	<LOQ	24	8.3	43.6	257.3
	AA-7	0.33	19.8		1.30	<LOQ	41.20	22.60	<LOQ	4.20	10.20	7.80	24	8.5	46.9	244.0
			MEAN		3.04	0.80	9.81	6.95	0.94	2.02	2.34	1.31				
			SD		3.69	1.41	14.76	7.89	1.42	2.22	3.70	2.88				

[a]Limit of quantification of deisopropyl atrazine was 0.20 µg/L. DIA was detected at only one site (AA-7) in Aug 2003 at a concentration of 0.5 µg/L.

[b]Limits of quantification (LOQ) for atrazine was 0.20 µg/L

[c]Limit of quantification for desethyl atrazine was 0.4 µg/L.

LOQ – limit of quantification. Site area – Surface area of collection site. Watershed area – Watershed area of collection site (ha) – Hectare. T – Temperature. DO - dissolved oxygen (mg/L). Cond. – Conductivity (µS/cm)

The report does not provide any information on how long frogs may have been kept in transport buckets prior to blood being drawn. Frogs in close proximity to one another for prolonged periods of time could potentially demonstrate blood-borne hormone levels.

Based on the information contained within the report, there were four collection periods, and up to 17 frogs were collected at each sample; therefore, there should be a maximum of 68 frogs per pond. Based on the total number of post-metamorphic frogs provided in **Table 43** (depicting Table 2 from report) of the study, only one of the NAA ponds yielded 68 frogs; the remaining two ponds yielded 15 and 12 frogs each. The number of post-metamorphic frogs collected at AA ponds ranged from 39 to 57. The report does not state whether the 13 frogs [discussed earlier] that had not completed metamorphosis were from the AA or NAA ponds.

Although the paper appears to focus on post-metamorphic animals, the stage of sexual development is not discussed in the study. Plasma estradiol and testosterone levels could be affected by the developmental stage of the animals. Since bullfrogs breed from February through July in permanent water bodies (http://www.fs.fed.us/r4/amphibians/bullfrog.htm), it is possible that some proportion of the animals in this study were in the process of breeding.

Table 43. Number of post-metamorphic bullfrogs of each sex collected from non-agricultural area (NAA) and agricultural area (AA) sites in southern Iowa for all four sampling periods in 2003. Duplicates Table 2 of the Smith *et al*. 2007 report.

Table 2: Number of post-metamorphic bullfrogs of each sex collected from non-agricultural area (NAA) and agricultural area (AA) sites in southern Iowa for all four sampling periods in 2003.

		Sex (n)		Sex ratio (%)	
Site	n	Males	Females	Males	Females
NAA-1	15	9	6	60	40
NAA-2	12	5	7	42	58
NAA-3	68	28	40	41	59
Total NAA	**95**	**42**	**53**	**44**	**56**
AA-1	47	27	20	57	43
AA-2	57	27	30	47	53
AA-3	41	25	16	61	39
AA-4	49	36	13	73	23
AA-5	53	20	33	38	62
AA-6	63	34	29	54	46
AA-7	39	24	15	62	38
Total AA	**345**	**193**	**152**	**56**	**44**
All sites	*444*	*235*	*209*	*53*	*47*

Although the report stated that 4 (0.9%) of the 444 bullfrogs had some observable physical abnormalities, the report does not state whether these frogs were collected from NAA or AA sites.

Although there was no statistical difference between the sex ratio of frogs collected from NAA and AA sites, based on the information presented in **Table 43** the sex ratio of all of the frogs collected at NAA sites (44:56 male:female) is the exact opposite of all of the frogs collected at AA sites (56:44). Arithmetic mean sex ratios, by collection site, calculated using the data provided in **Table 43,** are 56:43 (male:female) and 48:52 (male:female) for AA and NAA sites, respectively.

Figures depicting condition indices in the report are difficult to read; however, based on the figures alone, there appear to be potential differences between the female mean condition factors across sampling area and sampling period. However, no statistical significance is depicted in the figures.

The report noted that a number of smaller bullfrogs from both the AA (n=54) and NAA (n=8) sites had one or more gonads that weighed less than 0.001 mg and that these animals were not included in calculations involving gonadosomatic index. Therefore, statistics performed on GSI are potentially biased. The GSI data do emphasize though that frogs were in different stages of development across the entire sampling period.

The report concludes that there were no differences in fractional volume of spermatogonia, spermatocytes, or spermatozoa between sites or between seasons. Because only males sampled in August and September were included in the analysis, this time frame does not represent much of a change in season.

The 1.3% incidence of testicular oocytes reported by the authors was based on what is termed as an "independent" subsample of 80 males collected both NAA and AA sites. The report does not state whether equal numbers of males from each of the two sampling areas were included in the subsample; however, the incidence should have been expressed relative to the sampling site.

No statistical differences were noted between sampling sites for either plasma testosterone or estradiol in males; however, for estradiol at least, there was considerable variability in the males collected from AA sites. The coefficient of variability (standard deviation ÷ mean) was 95.9% and indicates that it is likely that the test had very little capability of detecting differences given the level of variability.

In general, there are a number of confounding effects that limit the utility of this study. The study only measured atrazine and two of its degradates (DEA and DIA) when there may have been a much broader spectrum of pesticides present. Since turf and residential uses surrounded two of the three reference (NAA) sampling sites and impervious surfaces associated with residential settings could increase the extent of runoff from any chemical applications, the potential movement of other pesticides into the NAA ponds is not adequately characterized. Additionally, the time in which animals were collected and then transported back to the lab for processing, the wide range of animal sizes and the protracted sampling period (5 months) of the study would have increased the number of animals at different stages of sexual development. As such, any effort to distinguish differences in plasma hormone levels and GSI would likely have been confounded by the high levels of variability. Although the intent of this study was to examine aspects of reproductive status in bullfrogs, the study appears to have focused on males and very little information is presented on females.

This paper represents the final report of a three-year study that was reviewed as an interim report[8] for the 2003 SAP. At that time, EPA indicated that the number of water samples collected may not provide a sufficient characterization of the exposure potential to atrazine. Additionally, EPA indicated that it was difficult to put the information collected on steroid hormone levels and the incidence of gonadal deformities into perspective since this information is not generally available for the bullfrog. The same limitations identified in the interim report still apply to the final published study as well.

8 Crabtree, C., E. E. Smith, J. A. Carr. 2003. Histology of the gonads and analysis of hormone levels in the native bullfrog (*Rana catesbeiana*) collected from agricultural areas in southern Iowa: pilot project. The Institute of Environmental and Human Studies, Texas Technical University, Lubbock, Texas. Sponsor: Syngenta Crop Protection, Inc., Identification Number ECORISK Number TTU-02 (EPA MRID No. 458677-05).

Gross, T. S. 2007. Characterization of atrazine exposures and potential effects for amphibians inhabiting sugarcane-dominated ecosystems in Florida. Final Report. Ecotoxicology Laboratory, University of Florida at Gainesville. Sponsor: Syngenta Crop Protection, Inc., 410 Swing Rd, P. O. Box 18300, Greensboro, 27419. MRID 472044-01.

Description and Results of Study

The objective of this study was to characterize the development of the Bidder's organ (rudimentary ovary) and gonads in cane toads (*Bufo marinus*) collected in sugarcane agricultural sites (Cane), non-sugarcane agricultural sites (Agric), and non-agricultural/urban sites (Urban) in southern Florida and to evaluate the potential association between chemical exposure and gonadal abnormalities

Adult toads (snout-vent length≥90 mm; weight≥50 g) of both sexes were collected as available from all sites during the spring (May 2003) and from all but two (non-cane agricultural) of the sites during July 2003. Water samples were collected for atrazine determinations from several canals/ditches at each of the two Cane sites, one of the 4 Agric and one of the two Urban sites. Samples were pooled from each site for each date of collection and were analyzed using an ELISA with a limit of detection (LOD) of 0.05 µg/L. Concentrations of atrazine in plasma were also determined using ELISA with an LOD of 0.1 µg/L; concentrations of estradiol and testosterone in plasma were determined using radioimmunoassay with a LOD of 28.6 and 27.3 pg/mL for estradiol and testosterone, respectively. Body weight (g), snout-vent length (SVL in mm), and coloration were recorded for each animal collected. Blood was collected from unanesthetized toads via cardiac puncture. Animals were sacrificed using methyltricainesulfonate (MS222). Gender was determined morphologically normal males (normal testes with undeveloped Bidder's organ), females (ovarian tissue; absence of testicular tissue), or males with developed Bidder's organ; developed Bidder's organ represented by an "oogenesis score" ≥2; **Table 44**). Gonads were removed and weighed. Body coloration was noted as female-like (mottled), male-like (solid coloration), or intergrade (gender distinction unclear). Forelimb nuptial pads were scored as female-like (absent), male-like (present), or integrade (unclear). Gonads from toads with testicular tissue were also examined histologically; histology was only intended to verify the presence of testicular tissue and the absence or presence of ovarian tissue in the testes. The ovarian stage of the Bidder's organ was evaluated using a dissecting scope

Table 44. Bidder's organ oocyte staging criteria.

Stage	Morphology	Size
Stage 1	Undeveloped oocytes	Oocyte diameter <0.3 mm
Stage 2	Oocytes translucent	Oocyte diameter 0.3 – 0.45 mm
Stage 3	Oocytes opaque and pigmented (yellow)	Oocyte diameter 0.45 – 0.6 mm
Stage 5	Oocytes darkly pigmented with clear animal pole	Oocyte diameter 1 – 1.2 mm
Stage 6	Oocytes dark	Oocyte diameter 1.2 – 1.4

According to the author, body weights between males and males with developed Bidder's organ did not differ statistically (**Table 45**).

Table 46 of the report summarizes mean ovarian stage, male Bidder's organ stage, and GSI for each of the sampling sites by season. The authors note in the caption to **Table 46** that there were no seasonal differences in reproductive status across sites and that there were no seasonal differences noted for SVL, body weight, gonad weight, GSI, stage, plasma sex steroid, or atrazine concentrations.

According to the report, though, atrazine concentrations in Cane sites ranged from <0.10 to 12.6 µg/L; for Agric sites, atrazine concentrations ranged from <0.10 to 0.78 µg/L; and for Urban sites, atrazine concentrations ranged from <0.10 to 1.19 µg/L. The greatest concentrations of atrazine were associated with the spring season and sugarcane agriculture.

The study examined organochlorine pesticide tissue residues only since this class of pesticides had historically been associated with sugarcane production and the compounds "normally bioaccumulate." No significant correlations were detected between the incidence of developed male Bidder's organ and organochlorine chemicals across or within each site. The authors note though that a number of other pesticides are utilized in the sugarcane collection sites.

Table 45. Summary of measurement endpoints from cane toads collected in South Florida based on Table 2 taken from Gross 2007.

Table 2. Summary of results for *Bufo marinus* from sugarcane agricultural sites (Cane); non-sugarcane agricultural sites (Agric); and from non-agricultural urban sites (Urban). Results are listed as mean ± SE). Body weight and lengths were similar between sites and land-use within each gender. Gonad weights and GSI were greater ($P<0.05$) for females compared to normal males and males with developed BO , while BO and normal males were similar, and land-use differences were not noted for body weight, length, gonadal weight, or GSI.

		Cane		Agric				Urban	
	Gender	Cane1	Cane2	Agric1	Agric2	Agric3	Agric4	Urban1	Urban2
Ovarian/ Bidder's Organ Stage	Male	1 ± 0.00	1 ± 0.00	1 ± 0.00	1 ± 0.00	1 ± 0.00	1 ± 0.00	1 ± 0.00	1 ± 0.00
	Female	3.56 ± 0.15	3.21 ± 0.18	3.00 ± 0.31	2.59 ± 0.21	3.57 ± 0.72	2.50 ± 0.50	4.00 ± 0.81	3.33 ± 0.31
	BO male	3.06 ± 0.14	3.22 ± 0.17	3.75 ± 0.32	3.00 ± 0.41	------	------	-----	3.43 ± 0.29
Body SVL (mm)	Male	119 ± 1.6	105 ± 1.9	104 ± 2.59	122 ± 4.3	115	91 ± 4.0	103 ± 2.9	111 ± 1.8
	Female	127 ± 1.4	112 ± 2.0	110 ± 3.49	128 ± 3.1	108 ± 6.6	97 ± 15.0	81 ± 16.9	115 ± 3.8
	BO male	119 ± 2.0	113 ± 2.5	119 ± 7.98	116 ± 9.7	--------	--------	--------	122 ± 6.8
Body weight (g)	Male	138 ± 4.8	91 ± 4.9	79 ± 6.26	132 ± 11.4	117	50 ± 5.6	75 ± 7.2	107 ± 5.1
	Female	178 ± 5.9	112 ± 6.2	100 ± 9.79	162 ± 12.1	104 ± 19.9	64 ± 26.5	110 ± 25.4	121 ± 10.7
	BO male	146 ± 8.3	113 ± 8.0	117 ± 22.6	116 ± 29.4	--------	--------	-------	141 ± 20.8
Gonad weight (g)	Male	0.48 ± 0.02	0.29 ± 0.02	0.25 ± 0.03	0.45 ± 0.05	0.33	0.20 ± 0.03	0.38 ± 0.04	0.39 ± 0.03
	Female	12.39 ± 1.25	8.16 ± 1.18	4.95 ± 1.57	6.38 ± 1.82	6.36 ± 2.32	2.14 ± 1.86	0.54 ± 0.33	8.62 ± 2.35
	BO male	0.51 ± 0.04	0.35 ± 0.07	0.41 ± 0.13	0.42 ± 0.13	--------	--------	--------	0.47 ± 0.08
GSI	Male	0.35 ± 0.01	0.32 ± 0.03	0.30 ± 0.02	0.35 ± 0.04	0.28	0.42 ± 0.07	0.35 ± 0.05	0.35 ± 0.02
	Female	5.86 ± 0.45	6.45 ± 1.09	3.76 ± 1.01	2.69 ± 0.54	6.05 ± 2.29	2.59 ± 1.85	1.57 ± 0.18	5.34 ± 1.23
	BO male	0.38 ± 0.03	0.33± 0.07	0.32 ± 0.5	0.34 ± 0.04	--------	--------	--------	0.36 ± 0.05
Plasma E2 (pg/ml)	Male	450 ± 39	350 ± 46	365 ± 72	189 ± 28	315	291 ± 64	322 ± 105	178 ± 19
	Female	1106 ± 51	1649 ± 76	881 ± 89	1026 ± 53	1088± 79	1400 ± 431	1316 ± 390	1055 ± 53
	BO male	587 ± 89	722 ± 148	436 ± 150	470 ± 279	--------	----------	--------	312 ± 127
Plasma T (pg/ml)	Male	1549 ± 81	1225 ± 52	1658 ± 137	1275 ± 111	1650	1634 ± 89	1743 ± 54	1714 ± 54
	Female	647 ± 57	282 ± 21	699 ± 120	392 ± 20	453 ± 23	575 ± 40	460 ± 115	339 ± 27
	BO male	1107 ± 115	866 ± 123	1188 ± 274	877 ± 203	--------	--------	--------	927 ± 204
Plasma atrazine (pg/ml)	Male	566 ± 80	939 ± 186	931 ± 193	167 ± 48	585	757 ± 505	848 ± 264	213 ± 32
	Female	635 ± 73	576 ± 117	692 ± 163	332 ± 108	239 ± 102	112 ± 23	914 ± 731	285 ± 116
	BO male	505 ± 242	95 ± 35	769 ± 464	357 ± 242	--------	--------	--------	8 ± 8

Plasma atrazine concentrations ranged from 0 to 6.55 ng/mL at Cane sites, 0 to 4.87 ng/mL at Agric sites, and 0 to 5.55 ng/mL at Urban sites. According to the author, the broad ranges in plasma atrazine concentrations reflect the lack of correlation between atrazine exposure and gonadal anomalies and/or plasma steroid concentrations.

According to the study, 95% of the females had normal mottled body coloration, and 98% were without the male-patterned forelimb nuptial pads. On average for "normal" males, 63 – 100% had forelimb nuptial pads, while normal coloration was exhibited by 10 to 75%. For males with developed Bidder's organ, the range of means across sampling sites was 50 – 92% for nuptial pads, while normal male coloration was exhibited by 17 – 75%.

The authors note in the study that coloration was not a reliable criterion for identifying males and that misidentification of gender using body coloration was most prevalent for animals exposed to sugarcane production.

Tables 47 and **48** summarize the gender of toads collected from the various sites. Males with developed Bidder's organ were more prevalent in sugarcane-growing regions; however, toads in general were also more prevalent (N=486) in sugarcane-growing areas. The lowest number of males with developed Bidder's organ was from Urban sites; however, the lowest number of frogs collected (N=145) was from Urban sites.

No intersex testes (testes with oocytes) were observed in the study.

Table 46. Summary of mean measurement endpoints from cane toads collect in southern Florida based on Table 3 taken from Gross 2007.

Table 3. Mean (± SE) GSI and ovarian/Bidder's organ oocyte stage for *Bufo marinus* collected from sugarcane agricultural sites (Cane); non-sugarcane agricultural sites (Agric); and from non-agricultural/urban (Urban) sites for each season collected. Note: the lack of seasonal differences in reproductive status for *B. marinus* across sites and within each land use in this study. Seasonal differences were also not noted for body length, weight, gonad weight, GSI, stage, plasma sex steroid hormones or plasma atrazine.

	Female (n=378)			Normal Male (n=351)			BO Male (n=87)		
Land-use Site	Ovarian Stage	GSI	N	Bidder's organ stage	GSI	N	Bidder's organ stage	GSI	N
Cane Spring									
Cane 1	3.98 ± 0.28	6.37 ± 0.90	47	1.0	0.375 ± 0.02	47	3.00 ± 0.31	0.405 ± 0.05	13
Cane 2	3.44 ± 0.28	6.73 ± 1.45	48	1.0	0.320 ± 0.03	51	3.06 ± 0.31	0.335 ± 0.09	18
Cane Summer									
Cane 1	**3.38 ± 0.18**	**5.63 ± 0.44**	**106**	**1.0**	**0.332 ± 0.03**	**57**	**3.13 ± 0.16**	**0.361 ± 0.07**	**23**
Cane 2	**2.95 ± 0.20**	**6.14 ± 0.69**	**42**	**1.0**	**0.308 ± 0.04**	**20**	**3.43 ± 0.20**	**0.321 ± 0.04**	**14**
Cane-Total	**3.43 ± 0.12**	**6.08 ± 0.43**	**243**	**1.0**	**0.337 ± 0.01**	**175**	**3.15 ± 0.11**	**0.354 ± 0.04**	**68**
Agric Spring									
Agric 1	2.75 ± 0.45	3.56 ± 1.43	20	1.0	0.307 ± 0.02	26	3.33 ± 0.42	0.290 ± 0.04	6
Agric 2	2.69 ± 0.26	2.55 ± 0.64	32	1.0	0.332 ± 0.04	12	3.00	0.278	1
Agric 3	3.57 ± 0.60	6.05 ± 2.29	7	1.0	0.279	1	-------	-------	0
Agric 4	2.52 ± 0.50	2.59 ± 1.85	2	1.0	0.423 ± 0.07	9	-------	-------	0
Agric Summer									
Agric 1	**3.28 ± 0.39**	**3.56 ± 1.35**	**18**	**1.0**	**0.290 ± 0.04**	**13**	**3.50 ± 0.50**	**0.403 ± 0.15**	**2**
Agric 2	**2.49 ± 0.34**	**2.91 ± 0.95**	**21**	**1.0**	**0.377 ± 0.07**	**12**	**3.00 ± 0.57**	**0.365 ± 0.04**	**3**
Agric 3	-------	-------	**0**	-------			-------		
Agric 4	-------	-------	**0**	-------	-------		-------	-------	**0**
Agric-Total	**2.81 ± 0.17**	**3.23 ± 0.50**	**100**	**1.0**	**0.333 ± 0.02**	**73**	**3.25 ± 0.25**	**0.327 ± 0.03**	**12**
Urban Spring									
Urban 1	4.0 ± 0.81	1.57 ± 0.18	2	1.0	0.383 ± 0.03	29	-------	-------	0
Urban 2	3.37 ± 0.45	5.37 ± 1.54	19	1.0	0.367 ± 0.03	37	3.33 ± 0.67	0.313 ± 0.08	3
Urban Summer									
Urban 1	-------	-------	**0**	-------	-------	**0**	-------	-------	**0**
Urban 2	**3.29 ± 0.41**	**5.31 ± 1.74**	**14**	**1.0**	**0.327 ± 0.02**	**37**	**3.50 ± 0.29**	**0.39 ± 0.04**	**4**
Urban-Total	**3.37 ± 0.29**	**5.13 ± 1.17**	**35**	**1.0**	**0.353 ± 0.01**	**103**	**3.43 ± 0.30**	**0.357 ± 0.04**	**7**

Table 47 (top) and 48 (bottom) representing gender and mean oocyte stage, respectively. Tables represent Tables 4 and 5 taken from Gross 2007.

Table 4. Summary of gender results for adult *Bufo marinus* collected from sugarcane agricultural sites (Cane), non-sugarcane agricultural sites (Agric) and non-agricultural urban (Urban) sites. Results are listed as the number of animals collected for each site and for each gender. Gender was assigned per macroscopic examination of gonads at sacrifice. The incidence of BO males (significant development of Bidder's organ as ovarian tissue) is also listed as the percent of total males collected and as a percent of the total population collected for each site and land-use. The incidence of BO males differed ($P<0.05$) with site and land-use.

Land-use/site	Female	Normal male	BO male	Percent of males BO	Percent of population BO	TOTAL
Cane1	153	104	36	26	12	293
Cane2	90	71	32	31	17	193
Cane-Total	**243**	**175**	**68**	**28**	**14**	**486**
Agric1	38	39	8	17	9	85
Agric2	53	24	4	14	5	81
Agric3	7	1	0	0	0	8
Agric4	2	9	0	0	0	11
Agric-Total	**100**	**73**	**12**	**14**	**6**	**185**
Urban1	2	29	0	0	0	31
Urban2	33	74	7	9	6	114
Urban-Total	**35**	**103**	**7**	**6**	**5**	**145**

Table 5. Mean oocyte stage for ovarian tissue in female and developed Bidder's organs in BO male *Bufo marinus* collected from sugarcane agricultural sites (Cane); non-sugarcane agricultural sites (Agric) and from non-agricultural/urban sites (Urban). Results are summarized as the number of animals collected and the percent of total animals collected (% of total). Differences were not noted between sites due to differences in land use, with an increased incidence ($P<0.05$) of developed Bidder's organ at Cane sites. However, the stage of oocyte development and distribution was similar with no differences between seasons and no difference between the right and left Bidder's organ.

Land-use Site	Female (n=351)							BO Males (87)						
	Ovarian (oocyte) Stage							Bidder's Organ (oocyte) Stage						
	N=	1	2	3	4	5	6	N=	1	2	3	4	5	6
Cane1	153	28	32	13	14	38	28	36	0	10	13	13	0	0
Cane2	90	19	20	9	16	17	9	32	0	10	7	13	2	0
Cane-Total	**243**	**47 (19%)**	**52 (21%)**	**22 (9%)**	**30 (12%)**	**55 (23%)**	**37 (15%)**	**68**	**0**	**20 (29%)**	**20 (29%)**	**26 (38%)**	**2 (3%)**	**0**
Agric1	38	11	8	4	6	3	6	8	0	1	4	2	1	0
Agric2	53	13	21	5	6	5	3	4	0	1	2	1	0	0
Agric3	7	1	2	0	1	2	1	0						
Agric4	2	0	1	1	0	0	0	0						
Agric-Total	100	**25 (25%)**	**32 (32%)**	**10 (10%)**	**13 (13%)**	**10 (10%)**	**10 (10%)**	**12**	**0**	**2 (18%)**	**6 (33%)**	**3 (17%)**	**1 (6%)**	**0**
Urban1	2	0	0	1	0	1	0	0						
Urban2	33	6	6	8	3	4	6	7	0	1	2	4	0	0
Urban-Total	35	**6 (18%)**	**6 (18%)**	**9 (27%)**	**3 (9%)**	**5 (15%)**	**6 (18%)**	**7**	**0**	**1 (14%)**	**2 (29%)**	**4 (57%)**	**0**	**0**

Analysis

It is not clear from the methods section why surface water samples were not collected from all the frog sampling sites.

The study has no obvious control and/or reference sites to which to compare absence of chemical exposure and Bidder's organ development. Animals were determined to be adults based on SVL and weight; however, no apparent effort was made to determine the chronological age of the animals. The mixed age of the animals tested and the resulting broad range of body weight and SVL likely contributed to the variability and limited the ability of the study to detect any possible differences. Where females are typically larger than male toads, this study was not able to distinguish a significant difference for either weight or SVL.

Although the methods section indicates that only adult toads were selected based on weight and SVL criteria of 60 g and 90 mm, respectively, 105 (13%) animals were identified in the data summary tables with weights less than 60 g. Toads, with weights as low as 12 g, were apparently included in the sample. It is unclear whether these smaller animals would be considered sexually mature adults; however, the 60-g weight criterion appears to be arbitrary to begin with.

It is unclear from the paper whether Bidder's organ has been studied sufficiently to determine the extent to which oocytes occur naturally. The stage of Bidder's organ development was gauged using a dissecting scope (presumably fitted with an ocular micrometer), but it is not known how the morphology was readily determined. It is also unclear whether staging was based on a single oocyte or whether more than one oocyte had to be evaluated to assign a developmental stage. Although Figure 1 of the report (not reproduced in this review) attempts to depict ovaries, testes, and developed/undeveloped Bidder's organ, the images are not clear and it is not readily apparent what distinguishes the various organ types. This problem may be partly caused by different levels of magnification used in the various sections. Staging of Bidder's organ depicted in Figure 4 of the report is difficult to determine since the sections are blurred and appear to be represented at different levels of magnification. Ideally, the sections should include a micrometer for gauging size.

The methods section does not define how gonadosomatic (GSI) index was calculated. Typically, the index is calculated by dividing the gonad weight by the body weight and

expressing the quotient as a percentage. However, it is unclear whether the Bidder's organ weight was included in the gonad weight or whether the Bidder's organ was dissected away from the gonad prior to weighing. Presumably, the GSI is based on the gonad plus Bidder's organ weight.

The presence/absence of nuptial pads depicted in Figure 2 of the report (not reproduced in this review) is also unclear since the objects appear to be out of focus.

Table 45 of the report summarizing measurement endpoint means (± standard errors) across sampling sites and genders does not depict whether means were statistically different. The caption states that gonad weights and GSI were greater ($p<0.05$) for females compared to normal males and males with developed Bidder's organ. Presumably, none of the other measurement endpoints were statistically different between gender and/or sampling areas. Presumably, **Table 45** is summarizing measurement endpoints across sampling periods as well and thus introduces considerable variability. In this case, the animals could be at different sizes and stages of development since one group was collected in late spring and the other was collected during mid-summer

Plasma atrazine concentrations ranged from 0 to 6.55 ng/mL at Cane sites, 0 to 4.87 ng/mL at Agric sites, and 0 to 5.55 ng/mL at Urban sites; mean plasma concentrations of atrazine in plasma [reported in **Table 45**] ranged from 0.008 – 0.939 ng/L. According to the author, the broad ranges in plasma atrazine concentrations reflect the lack of correlation between atrazine exposure and gonadal anomalies and/or plasma steroid concentrations. However, the level of detection for atrazine in plasma is reported as 0.1 μg/L (100 ng/L) and even the maximum reported concentrations were orders of magnitude below the LOD; therefore, plasma atrazine concentrations are suspect. Taking the plasma atrazine concentrations at face value would suggest that atrazine exposure is not substantially different between sites; however, the reported ranges of atrazine in water suggested that atrazine concentrations were higher in sugarcane-growing sites.

The LOD for estradiol and testosterone radioimmunoassay were 28.6 and 27.3 pg/mL, respectively; however, the report does not state what the level of quantification is for the assays. Since the method for collecting the animals is not stated in the methods section, it is unclear how long and under what conditions the animals were retained prior to processing. According to the protocol that accompanies the report, toads may have been retained for 3 to 5 days before

processing. These conditions could have markedly impacted plasma steroid concentrations. Since **Table 45** apparently summarizes measurement endpoints across sampling periods, it potentially introduces considerable variability into the plasma steroid levels since the animals would likely have been at different stages of development in late spring compared to mid-summer.

Table 46 of the report summarizes measurement endpoints by collection season and notes the lack of seasonal differences in reproductive status across sites and within each land use. Additionally seasonal differences were not noted for SVL, body weight, gonad weight, GSI, stage, plasma sex steroid or atrazine concentrations. The broad range of sizes and presumably ages of animals used in the study likely increased variability in the study and made it difficult to detect differences between sampling areas and/or seasons.

The number of animals collected at each site, as reported in **Table 47** of the report, ranged from 8 to 293. Toads were apparently most abundant in the cane-growing sites. This information combined with the fact that surface water was not sampled at all of the collection areas, presumably due to the lack of water, suggests that habitats differed markedly between land-use sites. This difference in the number of frogs collected may be indicative of poorer habitats unable to sustain larger populations of frogs; conversely, where larger numbers of frogs were collected, competition for available resources may have limited food availability. The apparent lack of similarity between the sampling sites likely confounded efforts to detect differences in body size and may have influenced the developmental stage of the animals.

The ratio of males to females [based on data contained in **Table 47**] ranged from 0.40 – 0.44, 0.13 – 0.81, 0.69 – 0.93 at the Cane, Agric and Urban sites, respectively, based on "normal males". However, according to the report, no testicular oocytes were observed in the study, therefore, the testes of males with developed Bidder's organs presumably were relatively normal. Thus, it is unclear whether the development of the Bidder's organ affected testicular function.

According to **Table 45**, mean plasma estradiol concentrations in males across sample sites ranged from 178 to 450 pg/mL; in females, plasma estradiol concentrations ranged from 881 – 1400 pg/mL. In males with developed Bidder's organs, estradiol concentrations ranged from 312 – 722 pg/mL. The authors did not report a significant difference in plasma estradiol concentrations; however, the concentrations reported for females appear to be roughly three times

higher than that reported for males. Mean plasma estradiol concentrations reported for males with developed Bidder's organs do not appear to be different from those reported for "normal" males. This suggests that the presence of oocytes in male Bidder's organs did not markedly influence plasma estradiol concentrations.

It is not possible to determine from this study whether Bidder's organs transition through a stage where any oocytes development occurs or whether they normally retain a rudimentary condition throughout the life of the toad. It is also unclear whether previously proposed mechanisms of up-regulation of aromatase leading to decreased levels of testosterone and increased levels of estradiol and subsequent development of testicular oocytes relates to potential effects on Bidder's organ. If it is proposed that Bidder's organ is a sensitive indicator of estrogenic activity or may in some way be indicative of up-regulation of aromatase, then it is unclear why testes are not affected. Also, it is unclear why development of Bidder's organ does not lead to increased production of estradiol since the development of oocytes would likely be dependent on increased concentrations of estradiol that would presumably lead to an increased incidence of testicular oocytes.

The authors state that misidentification of gender based on body coloration was most prevalent for animals exposed to sugarcane agriculture "and other sites and land/use types." The authors hypothesize that the differences in body coloration may be due to the presence of substances or environmental factors at the sugarcane sites which increased the incidence of female-like body coloration and the incidence of males developed Bidder's organ. However, according to **Table 45**, there was no difference in the incidence of males with developed Bidder's organ across sampling sites; therefore, it is unclear why the author implies that the effect may be due to substances/environmental factors at sugarcane sites as opposed to other sites as well. It is unclear whether body coloration may have been influenced by environmental conditions such as vegetation and/or substrate as the toads attempted to camouflage themselves from potential predators.

No raw data are provided on measured atrazine concentrations in water collected from the various sampling sites. Rather, Figure 5 of the report (not reproduced in this review) summarizes atrazine concentrations; however, the figure cannot be used to distinguish between Urban and Cane sites since both bars in the graph are given the same color/pattern. Because the methods section does not provide much detail on how water samples were collected, it is

uncertain whether the sampling sites were in close proximity to atrazine application sites and/or times. Additionally, the concentrations reported in the study represent atrazine concentrations at the time the toads were collected; however, no information is available regarding the levels of exposure to atrazine [and its degradates] during the aquatic-phase of the toads when they may have been more susceptible to water-borne contaminants. Additionally, given the broad range in the size and presumably age of the animals, the exposure history of the animals is uncertain. Finally, it is unclear whether cane toads remain in relatively small territories (fidelity) or whether they are likely to move over considerable differences. Therefore, the exposures depicted in the figure may not be representative of conditions when the frogs were at developmentally vulnerable stages. As stated previously, water samples were not collected at all of the toad collection sites so it is uncertain whether potential exposure is adequately characterized. The data presented in the report suggest that at the time the water samples were collected, atrazine concentrations in cane-growing areas appeared to range up to an order of magnitude higher than at the other two collection areas.

The study acknowledges that a number of pesticides are used in sugarcane production sites; however, only organochlorine compounds were measured. The authors noted that no correlation was found between the measured concentrations of organochlorine pesticides, though. Although Table 8 of the report lists mean concentrations for "cyclodiene pesticides", chlordanes, "DDT derivatives", DDE, endosulfan, PAH's methoxychlor, and toxaphene, the unit of measurement is not provided. Relative to other organochlorine pesticides measured, DDT and its degradate DDE were high; however, without an understanding of the measurement units, it is difficult to put the concentrations into any meaningful perspective. It is clear from the report though that sugarcane-growing sites appeared to have higher levels of chemicals than the other sampling sites and that across sampling sites there appears to be a mixture of chemicals. Although the study only focused on organochlorine chemicals, it is reasonable to believe that other chemicals may have been present. As stated earlier, though, the chemical residues documented in the study represent concentrations at some but not all of the collection sites and may not be reflective of chemical species/concentrations that may have been present when animals were in their aquatic-phase of development when the toads may be more sensitive to developmental perturbations.

A thorough analysis of the raw data provided in this report has not been conducted since the reviewer believes that the study is seriously compromised by potential sampling biases and

uncertainties. The few statistically significant effects reported in the study would be difficult to attribute to one particular factor. The study underscores the difficulty in conducting field studies and controlling the range of environmental and methodological factors that can influence the interpretation of the study. Additionally, the current study indicates that while the number of males with developed Bidder's organ was substantially higher in cane-growing areas and ranged on average across all of the males sampled from 0 to 31%, none of these males were found to have testicular oocytes, *i.e.*, the abnormality appeared to be confined to the Bidder's organ. Since Bidder's organ is typically vestigial, the effects of its development are uncertain; however, the author notes that Bidder's organ may undergo development as a natural component of the reproductive cycle. Given that the study animals exhibited a considerable range of sizes and were collected at two different periods during the year, it is possible that the differing extents of Bidder's organ development may have been due to differing stages of reproductive development.

APPENDIX 2 STATISTICAL ANALYSES SUMMARY TABLES

Table 2A: Envirnomental Chemistry Summary Data

ENVIRONMENTAL PARAMETERS (MEAN + STD ERROR) BY TREATMENT CONCENTRATION AT WLI

Obs	CONC	_TYPE_	_FREQ_	_STAT_	HARD	pH	NITRATE	NH3
1	0.00	0	40	N	40.000	40.0000	40.0000	40.0000
2	0.00	0	40	MIN	124.000	7.8000	0.0000	0.0000
3	0.00	0	40	MAX	144.000	8.3000	8.4000	2.9200
4	0.00	0	40	MEAN	136.200	8.0800	1.1975	0.2040
5	0.00	0	40	STD	4.479	0.1305	1.8515	0.4623
6	0.01	0	17	N	17.000	17.0000	17.0000	17.0000
7	0.01	0	17	MIN	130.000	7.8000	0.0000	0.0000
8	0.01	0	17	MAX	140.000	8.3000	8.4000	0.5900
9	0.01	0	17	MEAN	136.471	8.0471	1.3647	0.1682
10	0.01	0	17	STD	3.281	0.1375	2.1653	0.1895
11	0.10	0	22	N	22.000	22.0000	22.0000	22.0000
12	0.10	0	22	MIN	128.000	7.8000	0.0000	0.0000
13	0.10	0	22	MAX	140.000	8.3000	8.8000	0.5400
14	0.10	0	22	MEAN	136.364	8.0818	1.2136	0.1214
15	0.10	0	22	STD	3.416	0.1368	1.9273	0.1657
16	0.20	0	20	N	20.000	20.0000	20.0000	20.0000
17	0.20	0	20	MIN	130.000	7.8000	0.0000	0.0000
18	0.20	0	20	MAX	140.000	8.3000	6.2000	0.5700
19	0.20	0	20	MEAN	136.000	8.0750	1.1600	0.1835
20	0.20	0	20	STD	3.044	0.1517	1.6037	0.1775
21	1.00	0	20	N	20.000	20.0000	20.0000	20.0000
22	1.00	0	20	MIN	132.000	7.8000	0.0000	0.0000
23	1.00	0	20	MAX	144.000	8.3000	7.5000	0.6600
24	1.00	0	20	MEAN	136.800	8.0800	1.3750	0.1525
25	1.00	0	20	STD	2.931	0.1399	1.7926	0.1989
26	25.00	0	19	N	19.000	19.0000	19.0000	19.0000
27	25.00	0	19	MIN	132.000	7.8000	0.0000	0.0000
28	25.00	0	19	MAX	144.000	8.2000	7.9000	1.4100
29	25.00	0	19	MEAN	136.947	8.0526	1.2105	0.2089
30	25.00	0	19	STD	3.358	0.1219	1.8622	0.3251
31	100.00	0	22	N	22.000	22.0000	22.0000	22.0000
32	100.00	0	22	MIN	128.000	7.8000	0.0000	0.0000
33	100.00	0	22	MAX	140.000	8.2000	7.5000	1.0000
34	100.00	0	22	MEAN	135.455	8.0864	1.1318	0.1723
35	100.00	0	22	STD	3.277	0.1283	1.8287	0.2491

ENVIRONMENTAL PARAMETERS (MEAN + STD ERROR) BY TREATMENT CONCENTRATION AT IGB 20

Obs	CONC	_TYPE_	_FREQ_	_STAT_	HARD	ALK	COND	pH	NITRATE	NH3
1	0.00	0	44	N	44.000	44.000	44.000	44.0000	44.0000	44.0000
2	0.00	0	44	MIN	88.000	138.000	683.000	7.6700	0.0000	0.0000
3	0.00	0	44	MAX	112.000	180.000	793.000	8.4100	0.3500	0.5700
4	0.00	0	44	MEAN	97.273	154.523	768.318	8.0152	0.0741	0.0716
5	0.00	0	44	STD	5.069	11.595	24.061	0.1977	0.0600	0.1269
6	0.01	0	22	N	22.000	22.000	22.000	22.0000	22.0000	22.0000
7	0.01	0	22	MIN	90.000	143.000	712.000	7.5000	0.0000	0.0000
8	0.01	0	22	MAX	106.000	182.000	800.000	8.4000	1.0600	0.5400
9	0.01	0	22	MEAN	96.818	155.500	772.727	8.0318	0.1250	0.0695
10	0.01	0	22	STD	4.171	10.919	22.577	0.2197	0.2277	0.1259
11	0.10	0	22	N	22.000	22.000	22.000	22.0000	22.0000	22.0000
12	0.10	0	22	MIN	90.000	140.000	716.000	7.6700	0.0000	0.0000
13	0.10	0	22	MAX	108.000	176.000	796.000	8.3300	0.4000	0.6000
14	0.10	0	22	MEAN	97.091	154.727	773.182	8.0023	0.0855	0.0932
15	0.10	0	22	STD	4.927	10.806	18.420	0.1946	0.0899	0.1579
16	0.20	0	22	N	22.000	22.000	22.000	22.0000	22.0000	22.0000
17	0.20	0	22	MIN	88.000	143.000	726.000	7.7100	0.0000	0.0000
18	0.20	0	22	MAX	106.000	183.000	800.000	8.3300	0.3500	0.5500
19	0.20	0	22	MEAN	97.000	155.955	773.909	8.0200	0.0864	0.0705
20	0.20	0	22	STD	4.889	10.882	17.728	0.1898	0.0838	0.1266
21	1.00	0	22	N	22.000	22.000	22.000	22.0000	22.0000	22.0000

22	1.00	0	22	MIN	88.000	143.000	697.000	7.7600	0.0000	0.0000
23	1.00	0	22	MAX	108.000	182.000	800.000	8.3600	0.9700	0.6000
24	1.00	0	22	MEAN	96.545	155.364	770.682	8.0505	0.1168	0.0755
25	1.00	0	22	STD	5.527	11.043	23.414	0.2020	0.2044	0.1389
26	25.00	0	22	N	22.000	22.000	22.000	22.0000	22.0000	22.0000
27	25.00	0	22	MIN	90.000	142.000	687.000	7.7000	0.0000	0.0000
28	25.00	0	22	MAX	112.000	180.000	797.000	8.4000	0.6200	0.4100
29	25.00	0	22	MEAN	97.273	155.545	772.273	8.0395	0.0991	0.0595
30	25.00	0	22	STD	5.257	10.671	25.219	0.1960	0.1344	0.1004
31	100.00	0	22	N	22.000	22.000	22.000	22.0000	22.0000	22.0000
32	100.00	0	22	MIN	90.000	140.000	674.000	7.7700	0.0000	0.0000
33	100.00	0	22	MAX	106.000	182.000	799.000	8.4500	0.3100	0.3500
34	100.00	0	22	MEAN	96.545	155.545	773.682	8.0514	0.1050	0.0600
35	100.00	0	22	STD	4.543	11.023	25.214	0.1933	0.0962	0.0935

APPENDIX 2B
WIL GROSS MORPHOLOGY AND HISTOPATHOLOGY STATISTICAL ANALYSES

TABLE 2B.1. WIL GROSS - Tests for **Tank Cluster Effects**: Wilcoxon-Mann-Whitney Tests and Tippett's Minimum p-value Method

Index #	Characteristic	Sex	p-value	Significant or Not Significant
1	Tadpole mortality	-	0.9287	Not Significant
2	Failure to complete metamorphosis	-	1.0000	Not Significant
3	Age at completion of metamorphosis	F	0.5724	Not Significant
		M	0.5724	Not Significant
4	Body weight	F	0.7903	Not Significant
		M	0.3376	Not Significant
5	Snout-to-vent length	F	0.3376	Not Significant
		M	0.3376	Not Significant
6	Gonadal image area	F	0.1837	Not Significant
		M	0.3376	Not Significant
7	Intersex (gross observation)	-	1.0000	Not Significant
8	Mixed tissue types (gross observation)	-	0.9905	Not Significant
9	Gonadal adhesions	F	1.0000	Not Significant
		M	1.0000	Not Significant
10	Gonadal aplasia	F	0.9998	Not Significant
		M	1.0000	Not Significant
11	Gonadal segmental aplasia	F	0.9801	Not Significant
		M	0.4660	Not Significant
12	Gonadal bifurcation	F	1.0000	Not Significant
		M	1.0000	Not Significant
13	Gonadal deformity	F	0.6601	Not Significant
		M	1.0000	Not Significant
14	Gonadal displacement	F	1.0000	Not Significant
		M	0.9801	Not Significant
15	Fused gonads	F	0.9801	Not Significant
		M	1.0000	Not Significant
16	Gonadal hypertrophy	F	1.0000	Not Significant
		M	0.6601	Not Significant
17	Gonadal segmental hypertrophy	F	0.9801	Not Significant
		M	1.0000	Not Significant
18	Gonadal hypoplasia	F	0.9861	Not Significant
		M	0.9801	Not Significant
19	Gonadal segmental hypoplasia	F	0.7319	Not Significant

TABLE 2B.1. WIL GROSS - Tests for **Tank Cluster Effects**: Wilcoxon-Mann-Whitney Tests and Tippett's Minimum p-value Method

Index #	Characteristic	Sex	p-value	Significant or Not Significant
		M	0.9801	Not Significant
20	Gonadal translucence	F	0.5724	Not Significant
		M	0.6601	Not Significant
21	Gonadal segmental translucence	F	0.6601	Not Significant
		M	0.9612	Not Significant
22	Gonadal pearling	F	1.0000	Not Significant
		M	1.0000	Not Significant
23	Melanophores decreased in ovaries	F	1.0000	Not Significant
24	Internal melanophores in testes	M	0.9801	Not Significant
25	Frequency of males among just males and females	-	0.4660	Not Significant
26	Frequency of males	-	0.4660	Not Significant
28	Frequency of intersex	-	1.0000	Not Significant
29	Frequency of mixed sex	-	0.9720	Not Significant

TABLE 2B.2. WIL GROSS - Testing All Measurement-Based Tank Endpoints **for Normality and Homogeneity** of Variances

Index #	Characteristic	Sex	Analysis Group	p-value	S or NS	p-value	S or NS
3	Age at completion of metamorphosis	F	ATZ	0.9131	NS	0.6234	NS
			E2	0.5071	NS	0.4317	NS
		M	ATZ	0.8722	NS	0.0125	NS
			E2	0.4523	NS	0.5477	NS
4	Body weight	F	ATZ	0.9858	NS	0.9904	NS
			E2	0.7264	NS	0.0455	NS
		M	ATZ	0.2912	NS	0.7979	NS
			E2	0.1504	NS	0.1879	NS
5	Snout-to-vent length	F	ATZ	0.3174	NS	0.6171	NS
			E2	0.6432	NS	0.1087	NS
		M	ATZ	0.8691	NS	0.9383	NS
			E2	0.7644	NS	0.1648	NS
6	Gonadal image area	F	ATZ	0.4928	NS	0.4629	NS
			E2	0.0092	S	0.2087	NS
		M	ATZ	0.1281	NS	0.5213	NS
			E2	0.8393	NS	0.7889	NS

TABLE 2B.3. WIL GROSS - Testing Transformations of Mean Gonadal Image Area in Females for Normality and Homogeneity of Variances

Index #	Characteristic	Sex	Analysis Group	Power in Box-Cox Transformation	Shapiro-Wilk Test for Non-Normality: p-value	Levene's Test for Heterogeneity of Variances: p-value	Acceptability
6	Gonadal image area	F	E2	1.0	0.0092	0.2087	
				0.5	0.0149	0.2012	Acceptable

TABLE 2B.4. WIL GROSS - **E2 Comparison to Control**: Measurement-Based Tank Measures and Incidence-Based Measures Not Grouped by Sex

Index #	Characteristic	Test	Test Type	Sex	p-value	S or NS
1	Tadpole mortality	Wilcoxon-Mann-Whitney	1+	-	0.9333	NS
2	Failure to complete metamorphosis	Wilcoxon-Mann-Whitney	1+	-	1.0000	NS
3	Age at completion of metamorphosis	t-test	2	F	0.1082	NS
				M	0.0130	S
4	Body weight	t-test	2	F	0.6311	NS
				M	0.1638	NS
5	Snout-to-vent length	t-test	2	F	0.7896	NS
				M	0.2411	NS
6	Gonadal image area	t-test	2	F	0.6164	NS
				M	0.0167	S
7	Intersex (gross observation)	Wilcoxon-Mann-Whitney	1+	-	1.0000	NS
8	Mixed tissue types (gross observation)	Wilcoxon-Mann-Whitney	1+	-	0.0385	S
25	Frequency of males among just males and females	Wilcoxon-Mann-Whitney	1-	-	0.0003	S
26	Frequency of males	Wilcoxon-Mann-Whitney	1-	-	0.0003	S
28	Frequency of intersex	Wilcoxon-Mann-Whitney	1+	-	1.0000	NS
29	Frequency of mixed sex	Wilcoxon-Mann-Whitney	1+	-	0.0035	S

TABLE 2B.5. WIL GROSS - Test for **Tank Effects among E2 Males** and Control Males for Incidence-Based Measures Grouped by Sex: Chi-Square Test and Tippet's minimum p-value

Index #	Characteristic	p-value	Significant or Not Significant
9	Gonadal adhesions	1.0000	Not Significant
10	Gonadal aplasia	1.0000	Not Significant
11	Gonadal segmental aplasia	0.7248	Not Significant
12	Gonadal bifurcation	0.6381	Not Significant
13	Gonadal deformity	0.9768	Not Significant
14	Gonadal displacement	1.0000	Not Significant
15	Fused gonads	1.0000	Not Significant
16	Gonadal hypertrophy	0.9287	Not Significant
17	Gonadal segmental hypertrophy	0.9927	Not Significant
18	Gonadal hypoplasia	0.1504	Not Significant
19	Gonadal segmental hypoplasia	0.4860	Not Significant
20	Gonadal translucence	0.3020	Not Significant
21	Gonadal segmental translucence	0.7926	Not Significant
22	Gonadal pearling	0.8438	Not Significant
24	Internal melanophores in testes	0.6381	Not Significant

TABLE 2B.6. WIL GROSS - **Comparison of E2 Males to Control Males** for Incidence-Based Measures Grouped by Sex: Fisher's Exact Test

Index #	Characteristic	Test Type	p-value	Significant or Not Significant
9	Gonadal adhesions	1+	1.0000	Not Significant
10	Gonadal aplasia	1+	1.0000	Not Significant
11	Gonadal segmental aplasia	1+	0.0853	Not Significant
12	Gonadal bifurcation	1+	0.1907	Not Significant
13	Gonadal deformity	1+	1.0000	Not Significant
14	Gonadal displacement	1+	1.0000	Not Significant
15	Fused gonads	1+	1.0000	Not Significant
16	Gonadal hypertrophy	1+	0.4891	Not Significant
17	Gonadal segmental hypertrophy	1+	0.0775	Not Significant
18	Gonadal hypoplasia	1+	0.0187	Significant
19	Gonadal segmental hypoplasia	1+	0.1545	Not Significant
20	Gonadal translucence	1+	1.0000	Not Significant
21	Gonadal segmental translucence	1+	0.0671	Not Significant
22	Gonadal pearling	1+	0.0775	Not Significant
24	Internal melanophores in testes	1+	0.4339	Not Significant

TABLE 2B.7. WIL GROSS - **Comparison of E2 Females to Control Females** for Incidence-Based Measures Grouped by Sex: Wilcoxon-Mann-Whitney Test

Index #	Characteristic	Test Type	p-value	Significant or Not Significant
9	Gonadal adhesions	1+	1.0000	Not Significant
10	Gonadal aplasia	1+	1.0000	Not Significant
11	Gonadal segmental aplasia	1+	0.5000	Not Significant
12	Gonadal bifurcation	1+	1.0000	Not Significant
13	Gonadal deformity	1+	0.9000	Not Significant
14	Gonadal displacement	1+	1.0000	Not Significant
15	Fused gonads	1+	0.5000	Not Significant
16	Gonadal hypertrophy	1+	0.2333	Not Significant
17	Gonadal segmental hypertrophy	1+	1.0000	Not Significant
18	Gonadal hypoplasia	1+	0.2778	Not Significant
19	Gonadal segmental hypoplasia	1+	0.1974	Not Significant
20	Gonadal translucence	1+	0.1696	Not Significant
21	Gonadal segmental translucence	1+	0.0408	Significant
22	Gonadal pearling	1+	1.0000	Not Significant
23	Melanophores decreased in ovaries	1+	1.0000	Not Significant

TABLE 2B.8. WIL GROSS - **Atrazine Treatment Groups and Controls**: Measurement-Based Tank Measures and Incidence-Based Measures Not Grouped by Sex

Index #	Characteristic	Test	Test Type	Sex	p-value	Treatment-Control Differences
1	Tadpole mortality	Kruskal-Wallis	1+	-	0.2364	None Significant
2	Failure to complete metamorphosis	Kruskal-Wallis	1+	-	1.0000	None Significant
3	Age at completion of metamorphosis	One-Way ANOVA F-Test	2	F	0.1817	None Significant
				M	0.5385	None Significant
4	Body weight	One-Way ANOVA F-Test	2	F	0.4286	None Significant
				M	0.7617	None Significant
5	Snout-to-vent length	One-Way ANOVA F-Test	2	F	0.5099	None Significant
				M	0.9209	None Significant
6	Gonadal image area	One-Way ANOVA F-Test	2	F	0.9271	None Significant
				M	0.0418	Separate Testing Required
7	Intersex (gross observation)	Kruskal-Wallis	1+	-	1.0000	None Significant
8	Mixed tissue types (gross observation)	Kruskal-Wallis	1+	-	1.0000	None Significant
25	Frequency of males among just males and females	Kruskal-Wallis	1-	-	0.5229	None Significant
26	Frequency of males	Kruskal-Wallis	1-	-	0.5221	None Significant
28	Frequency of intersex	Kruskal-Wallis	1+	-	1.0000	None Significant
29	Frequency of mixed sex	Kruskal-Wallis	1+	-	1.0000	None Significant

TABLE 2B.9. WIL GROSS - Pairwise ANOVA contrast comparisons of the **mean gonadal image area** in the five atrazine male treatment groups with control **males**

Atrazine Dose (ppm)	Mean Gonadal Image Area	Test Type	p-value	Significant or Not Significant
0.0 (Controls)	0.839	-	-	-
0.01	0.876	2	0.1140	Not Significant
0.10	0.831	2	0.7189	Not Significant
1.00	0.836	2	0.9058	Not Significant
25.00	0.861	2	0.3574	Not Significant
100.00	0.897	2	0.0155	Significant

TABLE 2B.10. WIL GROSS - **Atrazine Treatment Groups and Controls**: Incidence-Based Measures Grouped by Sex: Kruskal-Wallis Test

Index #	Characteristic	Test Type	Sex	p-value	Treatment-Control Differences
9	Gonadal adhesions	1+	F	0.2569	None Significant
			M	1.0000	None Significant
10	Gonadal aplasia	1+	F	0.1937	None Significant
			M	1.0000	None Significant
11	Gonadal segmental aplasia	1+	F	0.7911	None Significant
			M	0.2144	None Significant
12	Gonadal bifurcation	1+	F	1.0000	None Significant
			M	0.3162	None Significant
13	Gonadal deformity	1+	F	0.2076	None Significant
			M	1.0000	None Significant
14	Gonadal displacement	1+	F	1.0000	None Significant
			M	0.0356	Separate Testing Required
15	Fused gonads	1+	F	0.1836	None Significant
			M	1.0000	None Significant
16	Gonadal hypertrophy	1+	F	0.5908	None Significant
			M	0.0774	Separate Testing Required
17	Gonadal segmental hypertrophy	1+	F	0.1492	None Significant
			M	0.3075	None Significant
18	Gonadal hypoplasia	1+	F	0.4439	None Significant
			M	0.0616	Separate Testing Required
19	Gonadal segmental hypoplasia	1+	F	0.6114	None Significant
			M	0.6332	None Significant
20	Gonadal translucence	1+	F	0.4227	None Significant
			M	0.9630	None Significant
21	Gonadal segmental translucence	1+	F	0.0178	Separate Testing Required
			M	0.0002	Separate Testing Required
22	Gonadal pearling	1+	F	1.0000	None Significant
			M	1.0000	None Significant
23	Melanophores decreased in ovaries	1+	F	1.0000	None Significant
24	Internal melanophores in testes	1+	M	0.5983	None Significant

TABLE 2B.11 WIL GROSS - Wilcoxon-Mann-Whitney comparisons of the percent with **displacement** in the five atrazine Male treatment groups with control **Males**

Atrazine Dose (ppm)	Mean Rank (Treatment) - Mean Rank (Control)	Test Type	p-value	Significant or Not Significant
0.01	0.000	1+	1.0000	Not Significant
0.10	2.000	1+	0.2333	Not Significant
1.00	3.214	1+	0.0769	Not Significant
25.00	1.000	1+	0.5000	Not Significant
100.00	0.000	1+	1.0000	Not Significant

TABLE 2B.12. WIL GROSS - Wilcoxon-Mann-Whitney comparisons of the percent with **hyperthrophy** in the five atrazine Male treatment groups with control **Males**

Atrazine Dose (ppm)	Mean Rank (Treatment) - Mean Rank (Control)	Test Type	p-value	Significant or Not Significant
0.01	-2.875	1+	0.9079	Not Significant
0.10	-4.250	1+	0.9872	Not Significant
1.00	-4.688	1+	1.0000	Not Significant
25.00	-4.000	1+	0.9797	Not Significant
100.00	-2.750	1+	0.9014	Not Significant

TABLE 2B.13. WIL GROSS - Wilcoxon-Mann-Whitney comparisons of the percent with **hypoplasia** in the five atrazine Male treatment groups with control **Males**

Atrazine Dose (ppm)	Mean Rank (Treatment) - Mean Rank (Control)	Test Type	p-value	Significant or Not Significant
0.01	0.000	1+	0.5718	Not Significant
0.10	5.500	1+	0.0060	Significant
1.00	0.804	1+	0.3026	Not Significant
25.00	3.250	1+	0.0725	Not Significant
100.00	1.250	1+	0.2846	Not Significant

TABLE 2B.14. WIL GROSS - Wilcoxon-Mann-Whitney comparisons of the percent with **segmental translucence** in the five atrazine Male treatment groups with control **Males**

Atrazine Dose (ppm)	Mean Rank (Treatment) - Mean Rank (Control)	Test Type	p-value	Significant or Not Significant
0.01	1.875	1+	0.2231	Not Significant
0.10	0.125	1+	0.5000	Not Significant
1.00	1.473	1+	0.2000	Not Significant
25.00	-1.000	1+	1.0000	Not Significant
100.00	6.750	1+	0.0013	Significant

TABLE 2B.15. WIL GROSS - Wilcoxon-Mann-Whitney comparisons of the percent with **segmental translucence** in the five atrazine Female treatment groups with control **Females**

Atrazine Dose (ppm)	Mean Rank (Treatment) - Mean Rank (Control)	Test Type	p-value	Significant or Not Significant
0.01	3.250	1+	0.0641	Not Significant
0.10	6.750	1+	0.0013	Significant
1.00	2.411	1+	0.1282	Not Significant
25.00	6.875	1+	0.0007	Significant
100.00	4.375	1+	0.0221	Significant

TABLE 2B.16. WIL GROSS –Atrazine Treatment Groups and Controls: **Trend Tests**

Index #	Characteristic	# of Atrazine Doses	High Dose (ppm)	Test Type	Sex	p-value	Significant or Not Significant
1	Tadpole mortality	5	100	1+	-	0.3324	Not Significant
2	Failure to complete metamorphosis	5	100	1+	-	0.1872	Not Significant
3	Age at completion of metamorphosis	5	100	2	F	0.6061	Not Significant
		5	100		M	0.8268	Not Significant
4	Body weight	5	100	2	F	0.5061	Not Significant
		5	100		M	0.4265	Not Significant
5	Snout-to-vent length	5	100	2	F	0.8579	Not Significant
		5	100		M	0.3597	Not Significant
6	Gonadal image area	5	100	2	F	0.8487	Not Significant
		5	100		M	0.1672	Not Significant
7	Intersex (gross observation)	5	100	1+	-	1.0000	Not Significant
8	Mixed tissue types (gross observation)	5	100	1+	-	1.0000	Not Significant
9	Gonadal adhesions	5	100	1+	F	>0.9915	Not Significant
		5	100		M	>0.6600	Not Significant
10	Gonadal aplasia	5	100	1+	F	0.2507	Not Significant
		5	100		M	1.0000	Not Significant
11	Gonadal segmental aplasia	5	100	1+	F	>0.8420	Not Significant
		5	100		M	>0.9038	Not Significant
12	Gonadal bifurcation	5	100	1+	F	0.3394	Not Significant
		5	100		M	0.3478	Not Significant
13	Gonadal deformity	5	100	1+	F	0.4759	Not Significant
		5	100		M	>0.5136	Not Significant
14	Gonadal displacement	5	100	1+	F	>0.6716	Not Significant
		5	100		M	0.3025	Not Significant
15	Fused gonads	5	100	1+	F	0.0685	Not Significant
		5	100		M	>0.4834	Not Significant
16	Gonadal hypertrophy	5	100	1+	F	0.1629	Not Significant
		5	100		M	>0.9674	Not Significant
17	Gonadal segmental hypertrophy	5	100	1+	F	>0.5834	Not Significant
		5	100		M	0.1323	Not Significant
18	Gonadal hypoplasia	5	100	1+	F	0.3635	Not Significant
		5	100		M	0.1877	Not Significant

TABLE 2B.16. WIL GROSS –Atrazine Treatment Groups and Controls: **Trend Tests**

Index #	Characteristic	# of Atrazine Doses	High Dose (ppm)	Test Type	Sex	p-value	Significant or Not Significant
19	Gonadal segmental hypoplasia	5	100	1+	F	0.3157	Not Significant
		5	100		M	>0.6469	Not Significant
20	Gonadal translucence	5	100	1+	F	0.1172	Not Significant
		5	100		M	>0.5197	Not Significant
21	Gonadal segmental translucence	5	100	1+	F	0.0210	Significant
		4	25			0.0020	Significant
		3	1			0.0250	Significant
		2	0.1			0.0009	Significant
		1	0.01			0.0670	Not Significant
		5	100		M	0.0086	Significant
		4	25			>0.7156	Not Significant
22	Gonadal pearling	5	100	1+	F	1.0000	Not Significant
		5	100		M	0.3369	Not Significant
23	Melanophores decreased in ovaries	5	100	1+	F	>0.5242	Not Significant
24	Internal melanophores in testes	5	100	1+	M	>0.9061	Not Significant
25	Frequency of males among just males and females	5	100	1-	-	0.0795	Not Significant
26	Frequency of males	5	100	1-	-	0.0754	Not Significant
28	Frequency of intersex	5	100	1+	-	1.0000	Not Significant
29	Frequency of mixed sex	5	100	1+	-	1.0000	Not Significant

TABLE 2B.17. WIL EPL- Tests for **Tank Cluster Effects**: Wilcoxon-Mann-Whitney Tests and Tippett's Minimum p-value Method

Index #	Characteristic	Sex	p-value	Significant or Not Significant
1	Segmental hypoplasia (Grade>0)	F	0.9801	Not Significant
		M	0.3376	Not Significant
	Segmental hypoplasia (Grade>1)	F	1.0000	Not Significant
		M	0.9998	Not Significant
2	Segmental aplasia (Grade>0)	F	1.0000	Not Significant
		M	0.9801	Not Significant
	Segmental aplasia (Grade>1)	F	1.0000	Not Significant
		M	0.6601	Not Significant
3	Decreased germ cells in gonad (Grade>0)	F	0.5724	Not Significant
		M	0.8374	Not Significant
	Decreased germ cells in gonad (Grade>1)	F	0.9905	Not Significant
		M	0.5724	Not Significant
4	Dilated testis tubules (Grade>0)	M	0.6601	Not Significant
	Dilated testis tubules (Grade>1)	M	0.6601	Not Significant
5	Dividing gonocytes in testes (Grade>0)	M	0.7903	Not Significant
	Dividing gonocytes in testes (Grade>1)	M	0.9998	Not Significant
6	Internal melanophores in testis (Grade>0)	M	0.5724	Not Significant
	Internal melanophores in testis (Grade>1)	M	0.3376	Not Significant
7	Decreased ovarian pigmentation (Grade>0)	F	1.0000	Not Significant
	Decreased ovarian pigmentation (Grade>1)	F	1.0000	Not Significant
8	Decreased ovarian cavity size (Grade>0)	F	0.9471	Not Significant
	Decreased ovarian cavity size (Grade>1)	F	0.3376	Not Significant
9	Increased ovarian cavity size (Grade>0)	F	0.9801	Not Significant
	Increased ovarian cavity size (Grade>1)	F	0.9998	Not Significant
10	Ovarian interstitial fibrosis (Grade>0)	F	0.1837	Not Significant
	Ovarian interstitial fibrosis (Grade>1)	F	0.9612	Not Significant
11	Mononuclear cell infiltrates in ovary (Grade>0)	F	0.1837	Not Significant
	Mononuclear cell infiltrates in ovary (Grade>1)	F	0.9801	Not Significant
12	One or more immature ovaries	F	0.4660	Not Significant
	One or more unable to stage ovaries	F	0.5724	Not Significant
13	Fused kidneys (Grade>0)	F	0.9471	Not Significant
		M	0.7319	Not Significant
	Fused kidneys (Grade>1)	F	1.0000	Not Significant
		M	0.9801	Not Significant

TABLE 2B.17. WIL EPL- Tests for **Tank Cluster Effects**: Wilcoxon-Mann-Whitney Tests and Tippett's Minimum p-value Method

Index #	Characteristic	Sex	p-value	Significant or Not Significant
14	Mineralization (Grade>0)	F	0.9287	Not Significant
		M	0.9051	Not Significant
	Mineralization (Grade>1)	F	1.0000	Not Significant
		M	1.0000	Not Significant
15	Other renal diagnoses (Grade>0)	F	0.9801	Not Significant
		M	0.9801	Not Significant
	Other renal diagnoses (Grade>1)	F	0.9801	Not Significant
		M	1.0000	Not Significant

TABLE 2B.18. WIL EPL - Test for **Tank Effects among E2 Males** and Control Males for Incidence-Based Measures Grouped by Sex: Chi-Square Test and Tippet's minimum p-value

Index #	Characteristic	p-value	Significant or Not Significant
1	Segmental hypoplasia (Grade>0)	0.8742	Not Significant
	Segmental hypoplasia (Grade>1)	0.7926	Not Significant
2	Segmental aplasia (Grade>0)	0.0876	Not Significant
	Segmental aplasia (Grade>1)	0.4810	Not Significant
3	Decreased germ cells in gonad (Grade>0)	0.7973	Not Significant
	Decreased germ cells in gonad (Grade>1)	0.7489	Not Significant
4	Dilated testis tubules (Grade>0)	0.4595	Not Significant
	Dilated testis tubules (Grade>1)	0.9768	Not Significant
5	Dividing gonocytes in testes (Grade>0)	0.4564	Not Significant
	Dividing gonocytes in testes (Grade>1)	0.1773	Not Significant
6	Internal melanophores in testis (Grade>0)	0.1779	Not Significant
	Internal melanophores in testis (Grade>1)	0.7102	Not Significant
13	Fused kidneys (Grade>0)	0.6654	Not Significant
	Fused kidneys (Grade>1)	1.0000	Not Significant
14	Mineralization (Grade>0)	0.1080	Not Significant
	Mineralization (Grade>1)	0.1080	Not Significant
15	Other renal diagnoses (Grade>0)	0.9375	Not Significant
	Other renal diagnoses (Grade>1)	0.9375	Not Significant

TABLE 2B.19. WIL EPL -Comparison of **E2 Males to Control Males** for Incidence-Based Measures Grouped by Sex: Fisher's Exact Test

Index #	Characteristic	Test Type	p-value	Significant or Not Significant
1	Segmental hypoplasia (Grade>0)	1+	0.2164	Not Significant
	Segmental hypoplasia (Grade>1)	1+	0.1349	Not Significant
2	Segmental aplasia (Grade>0)	1+	0.2220	Not Significant
	Segmental aplasia (Grade>1)	1+	0.0671	Not Significant
3	Decreased germ cells in gonad (Grade>0)	1+	0.9550	Not Significant
	Decreased germ cells in gonad (Grade>1)	1+	0.2519	Not Significant
4	Dilated testis tubules (Grade>0)	1+	<0.0001	Significant
	Dilated testis tubules (Grade>1)	1+	<0.0001	Significant
5	Dividing gonocytes in testes (Grade>0)	1+	<0.0001	Significant
	Dividing gonocytes in testes (Grade>1)	1+	0.0014	Significant
6	Internal melanophores in testis (Grade>0)	1+	0.0015	Significant
	Internal melanophores in testis (Grade>1)	1+	0.0001	Significant
13	Fused kidneys (Grade>0)	1+	0.9278	Not Significant
	Fused kidneys (Grade>1)	1+	1.0000	Not Significant
14	Mineralization (Grade>0)	2	0.2806	Not Significant
	Mineralization (Grade>1)	2	0.2812	Not Significant
15	Other renal diagnoses (Grade>0)	2	0.2812	Not Significant
	Other renal diagnoses (Grade>1)	2	0.2812	Not Significant

TABLE 2B.20. WIL EPL -**Comparison of E2 Females to Control Females** for Incidence-Based Measures Grouped by Sex: Wilcoxon-Mann-Whitney Test

Index #	Characteristic	Test Type	p-value	Significant or Not Significant
1	Segmental hypoplasia (Grade>0)	1+	1.0000	Not Significant
	Segmental hypoplasia (Grade>1)	1+	1.0000	Not Significant
2	Segmental aplasia (Grade>0)	1+	1.0000	Not Significant
	Segmental aplasia (Grade>1)	1+	1.0000	Not Significant
3	Decreased germ cells in gonad (Grade>0)	1+	0.1596	Not Significant
	Decreased germ cells in gonad (Grade>1)	1+	0.1337	Not Significant
7	Decreased ovarian pigmentation (Grade>0)	1+	1.0000	Not Significant
	Decreased ovarian pigmentation (Grade>1)	1+	1.0000	Not Significant
8	Decreased ovarian cavity size (Grade>0)	1+	0.0835	Not Significant
	Decreased ovarian cavity size (Grade>1)	1+	0.0384	Significant
9	Increased ovarian cavity size (Grade>0)	1+	0.0035	Significant
	Increased ovarian cavity size (Grade>1)	1+	0.1000	Not Significant
10	Ovarian interstitial fibrosis (Grade>0)	1+	0.3679	Not Significant
	Ovarian interstitial fibrosis (Grade>1)	1+	0.2333	Not Significant
11	Mononuclear cell infiltrates in ovary (Grade>0)	1+	0.4490	Not Significant
	Mononuclear cell infiltrates in ovary (Grade>1)	1+	0.7667	Not Significant
12	One or more immature ovaries	1+	0.5517	Not Significant
	One or more unable to stage ovaries	2	0.3943	Not Significant
13	Fused kidneys (Grade>0)	1+	0.4086	Not Significant
	Fused kidneys (Grade>1)	1+	1.0000	Not Significant
14	Mineralization (Grade>0)	2	0.8982	Not Significant
	Mineralization (Grade>1)	2	1.0000	Not Significant
15	Other renal diagnoses (Grade>0)	2	0.2000	Not Significant
	Other renal diagnoses (Grade>1)	2	1.0000	Not Significant

TABLE 2B.21. WIL EPL -**Atrazine Treatment Groups and Controls**: Incidence-Based Measures Grouped by Sex: Kruskal-Wallis Test

Index #	Characteristic	Test Type	Sex	p-value	Treatment-Control Differences
1	Segmental hypoplasia (Grade>0)	1+	F	0.7039	None Significant
			M	0.0283	Separate Testing Required
	Segmental hypoplasia (Grade>1)	1+	F	1.0000	None Significant
			M	0.2136	None Significant
2	Segmental aplasia (Grade>0)	1+	F	0.8989	None Significant
			M	0.0479	Separate Testing Required
	Segmental aplasia (Grade>1)	1+	F	1.0000	None Significant
			M	0.1369	None Significant
3	Decreased germ cells in gonad (Grade>0)	1+	F	0.8278	None Significant
			M	0.8866	None Significant
	Decreased germ cells in gonad (Grade>1)	1+	F	0.4914	None Significant
			M	0.3594	None Significant
4	Dilated testis tubules (Grade>0)	1+	M	0.0388	Separate Testing Required
	Dilated testis tubules (Grade>1)	1+	M	0.4825	None Significant
5	Dividing gonocytes in testes (Grade>0)	1+	M	0.1969	None Significant
	Dividing gonocytes in testes (Grade>1)	1+	M	0.0607	Separate Testing Required
6	Internal melanophores in testis (Grade>0)	1+	M	0.3658	None Significant
	Internal melanophores in testis (Grade>1)	1+	M	0.8763	None Significant
7	Decreased ovarian pigmentation (Grade>0)	1+	F	1.0000	None Significant
	Decreased ovarian pigmentation (Grade>1)	1+	F	1.0000	None Significant
8	Decreased ovarian cavity size (Grade>0)	1+	F	0.2186	None Significant
	Decreased ovarian cavity size (Grade>1)	1+	F	0.6373	None Significant
9	Increased ovarian cavity size (Grade>0)	1+	F	0.4226	None Significant
	Increased ovarian cavity size (Grade>1)	1+	F	1.0000	None Significant
10	Ovarian interstitial fibrosis (Grade>0)	1+	F	0.2500	None Significant
	Ovarian interstitial fibrosis (Grade>1)	1+	F	0.1937	None Significant
11	Mononuclear cell infiltrates in ovary (Grade>0)	1+	F	0.7989	None Significant
	Mononuclear cell infiltrates in ovary (Grade>1)	1+	F	0.1527	None Significant
12	One or more immature ovaries	1+	F	0.0403	Separate Testing Required
	One or more unable to stage ovaries	2	F	0.8579	None Significant
13	Fused kidneys (Grade>0)	1+	F	0.7913	None Significant
			M	0.0140	Separate Testing Required

TABLE 2B.21. WIL EPL -**Atrazine Treatment Groups and Controls**: Incidence-Based Measures Grouped by Sex: Kruskal-Wallis Test

Index #	Characteristic	Test Type	Sex	p-value	Treatment-Control Differences
	Fused kidneys (Grade>1)	1+	F	1.0000	None Significant
			M	0.1467	None Significant
14	Mineralization (Grade>0)	2	F	0.5906	None Significant
			M	0.6677	None Significant
	Mineralization (Grade>1)	2	F	1.0000	None Significant
			M	1.0000	None Significant
15	Other renal diagnoses (Grade>0)	2	F	0.1175	None Significant
			M	0.3319	None Significant
	Other renal diagnoses (Grade>1)	2	F	0.1997	None Significant
			M	0.2783	None Significant

TABLE 2B.22. WIL EPL -Percent of **Males with Segmental Hypoplasia (Grade>0)**: Wilcoxon-Mann-Whitney Comparisons of the Five Atrazine Treatment Groups with Controls

Atrazine Dose (ppm)	Mean Rank (Treatment) - Mean Rank (Control)	Test Type	p-value	Significant or Not Significant
0.01	-6.125	1+	0.9970	Not Significant
0.10	-4.375	1+	0.9705	Not Significant
1.00	-6.964	1+	0.9997	Not Significant
25.00	-4.750	1+	0.9796	Not Significant
100.00	-5.375	1+	0.9904	Not Significant

TABLE 2B.23. WIL EPL -Percent of **Males with Segmental Aplasia (Grade>0)**: Wilcoxon-Mann-Whitney Comparisons of the Five Atrazine Treatment Groups with Controls

Atrazine Dose (ppm)	Mean Rank (Treatment) - Mean Rank (Control)	Test Type	p-value	Significant or Not Significant
0.01	0.500	1+	0.4641	Not Significant
0.10	3.250	1+	0.0790	Not Significant
1.00	-0.268	1+	0.6615	Not Significant
25.00	3.375	1+	0.0725	Not Significant
100.00	-0.500	1+	0.7154	Not Significant

TABLE 2B.24. WIL EPL -Percent **of Males with Dilated Testis Tubules (Grade>0**): Wilcoxon-Mann-Whitney Comparisons of the Five Atrazine Treatment Groups with Controls

Atrazine Dose (ppm)	Mean Rank (Treatment) - Mean Rank (Control)	Test Type	p-value	Significant or Not Significant
0.01	-2.000	1+	0.8054	Not Significant
0.10	-2.625	1+	0.8718	Not Significant
1.00	-4.018	1+	0.9627	Not Significant
25.00	-3.375	1+	0.9298	Not Significant
100.00	3.500	1+	0.0758	Not Significant

TABLE 2B.25. WIL EPL -Percent of **Males with Dividing Gonocytes** in Testes (Grade>1): Wilcoxon-Mann-Whitney Comparisons of the Five Atrazine Treatment Groups with Controls

Atrazine Dose (ppm)	Mean Rank (Treatment) - Mean Rank (Control)	Test Type	p-value	Significant or Not Significant
0.01	1.000	1+	0.5000	Not Significant
0.10	3.000	1+	0.1000	Not Significant
1.00	0.000	1+	1.0000	Not Significant
25.00	1.000	1+	0.5000	Not Significant
100.00	0.000	1+	1.0000	Not Significant

TABLE 2B.26. WIL EPL - Percent of **Females with One or More Immature Ovaries**: Wilcoxon-Mann-Whitney Comparisons of the Five Atrazine Treatment Groups with Controls

Atrazine Dose (ppm)	Mean Rank (Treatment) - Mean Rank (Control)	Test Type	p-value	Significant or Not Significant
0.01	-3.125	1+	0.9615	Not Significant
0.10	-1.500	1+	0.7741	Not Significant
1.00	-3.214	1+	0.9814	Not Significant
25.00	0.625	1+	0.4021	Not Significant
100.00	-4.000	1+	1.0000	Not Significant

TABLE 2B.27. WIL EPL –Percent of **Males with Fused Kidneys (Grade >0**): Wilcoxon-Mann-Whitney Comparisons of the Five Atrazine Treatment Groups with Controls

Atrazine Dose (ppm)	Mean Rank (Treatment) - Mean Rank (Control)	Test Type	p-value	Significant or Not Significant
0.01	-1.375	1+	0.7280	Not Significant
0.10	-1.625	1+	0.7713	Not Significant
1.00	4.286	1+	0.0317	Significant
25.00	1.875	1+	0.2303	Not Significant
100.00	3.875	1+	0.0552	Not Significant

TABLE 2B.27. WIL EPL –Percent of **Males with Fused Kidneys (Grade >0**): Wilcoxon-Mann-Whitney Comparisons of the Five Atrazine Treatment Groups with Controls

Index #	Characteristic	# of Atrazine Doses	High Dose (ppm)	Test Type	Sex	p-value	Significant or Not Significant
1	Segmental hypoplasia (Grade>0)	5	100	1+	F	>0.7222	Not Significant
		5	100		M	>0.9253	Not Significant
	Segmental hypoplasia (Grade>1)	5	100	1+	F	>0.9494	Not Significant
		5	100		M	>0.5204	Not Significant
2	Segmental aplasia (Grade>0)	5	100	1+	F	>0.8841	Not Significant
		5	100		M	0.4652	Not Significant
	Segmental aplasia (Grade>1)	5	100	1+	F	>0.9170	Not Significant
		5	100		M	>0.6022	Not Significant
3	Decreased germ cells in gonad (Grade>0)	5	100	1+	F	0.1282	Not Significant
		5	100		M	>0.5949	Not Significant
	Decreased germ cells in gonad (Grade>1)	5	100	1+	F	0.1822	Not Significant
		5	100		M	0.1549	Not Significant
4	Dilated testis tubules (Grade>0)	5	100	1+	M	0.2338	Not Significant
	Dilated testis tubules (Grade>1)	5	100	1+	M	0.1440	Not Significant
5	Dividing gonocytes in testes (Grade>0)	5	100	1+	M	0.1327	Not Significant
	Dividing gonocytes in testes (Grade>1)	5	100	1+	M	>0.6393	Not Significant
6	Internal melanophores in testis (Grade>0)	5	100	1+	M	>0.8361	Not Significant
	Internal melanophores in testis (Grade>1)	5	100	1+	M	>0.7423	Not Significant
7	Decreased ovarian pigmentation (Grade>0)	5	100	1+	F	0.3348	Not Significant
	Decreased ovarian pigmentation (Grade>1)	5	100	1+	F	0.3412	Not Significant
8	Decreased ovarian cavity size (Grade>0)	5	100	1+	F	0.2190	Not Significant
	Decreased ovarian cavity size (Grade>1)	5	100	1+	F	>0.6490	Not Significant
9	Increased ovarian cavity size (Grade>0)	5	100	1+	F	0.4567	Not Significant
	Increased ovarian cavity size (Grade>1)	5	100	1+	F	0.1756	Not Significant
10	Ovarian interstitial fibrosis (Grade>0)	5	100	1+	F	0.0462	Significant
		4	25			0.2215	Not Significant
	Ovarian interstitial fibrosis (Grade>1)	5	100	1+	F	0.2268	Not Significant

TABLE 2B.27. WIL EPL –Percent of **Males with Fused Kidneys (Grade >0**): Wilcoxon-Mann-Whitney Comparisons of the Five Atrazine Treatment Groups with Controls

Index #	Characteristic	# of Atrazine Doses	High Dose (ppm)	Test Type	Sex	p-value	Significant or Not Significant
11	Mononuclear cell infiltrates in ovary (Grade>0)	5	100	1+	F	>0.7775	Not Significant
	Mononuclear cell infiltrates in ovary (Grade>1)	5	100	1+	F	0.4823	Not Significant
12	One or more immature ovaries	5	100	1+	F	>0.8660	Not Significant
	One or more unable to stage ovaries	5	100	2	F	0.7612	Not Significant
13	Fused kidneys (Grade>0)	5	100	1+	F	0.1413	Not Significant
		5	100		M	0.0082	Significant
		4	25			0.0444	Significant
		3	1			0.0566	Not Significant
	Fused kidneys (Grade>1)	5	100	1+	F	1.0000	Not Significant
		5	100		M	0.4890	Not Significant
14	Mineralization (Grade>0)	5	100	2	F	0.4384	Not Significant
		5	100		M	0.4090	Not Significant
	Mineralization (Grade>1)	5	100	2	F	0.6458	Not Significant
		5	100		M	0.3414	Not Significant
15	Other renal diagnoses (Grade>0)	5	100	2	F	0.4974	Not Significant
		5	100		M	0.2604	Not Significant
	Other renal diagnoses (Grade>1)	5	100	2	F	0.6643	Not Significant
		5	100		M	0.2989	Not Significant

APPENDIX 2C
IGB GROSS MORPHOLOGY AND HISTOPATHOLOGY STATISTICAL ANALYSES

TABLE 2C.1. IGB GROSS-Tests for **Tank Cluster Effects**: Wilcoxon-Mann-Whitney Tests and Tippett's Minimum p-value Method

Index #	Characteristic	Sex	p-value	Significant or Not Significant
1	Tadpole mortality	-	0.9612	Not Significant
2	Failure to complete metamorphosis	-	1.0000	Not Significant
3	Age at completion of metamorphosis	F	0.1837	Not Significant
		M	0.9905	Not Significant
4	Body weight	F	0.7838	Not Significant
		M	0.7903	Not Significant
5	Snout-to-vent length	F	0.7903	Not Significant
		M	0.3376	Not Significant
6	Gonadal image area	F	0.9471	Not Significant
		M	0.5724	Not Significant
7	Intersex (gross observation)	-	1.0000	Not Significant
8	Mixed tissue types (gross observation)	-	0.7379	Not Significant
9	Gonadal adhesions	F	1.0000	Not Significant
		M	1.0000	Not Significant
10	Gonadal aplasia	F	1.0000	Not Significant
		M	1.0000	Not Significant
11	Gonadal segmental aplasia	F	1.0000	Not Significant
		M	0.6601	Not Significant
12	Gonadal bifurcation	F	1.0000	Not Significant
		M	1.0000	Not Significant
13	Gonadal deformity	F	0.1837	Not Significant
		M	1.0000	Not Significant
14	Gonadal displacement	F	0.9998	Not Significant
		M	0.6601	Not Significant
15	Fused gonads	F	0.9801	Not Significant
		M	1.0000	Not Significant
16	Gonadal hypertrophy	F	0.9471	Not Significant
		M	0.7903	Not Significant
17	Gonadal segmental hypertrophy	F	1.0000	Not Significant
		M	1.0000	Not Significant
18	Gonadal hypoplasia	F	0.3376	Not Significant
		M	0.2792	Not Significant

TABLE 2C.1. IGB GROSS-Tests for **Tank Cluster Effects**: Wilcoxon-Mann-Whitney Tests and Tippett's Minimum p-value Method

Index #	Characteristic	Sex	p-value	Significant or Not Significant
19	Gonadal segmental hypoplasia	F	0.5724	Not Significant
		M	1.0000	Not Significant
20	Gonadal translucence	F	0.7779	Not Significant
		M	0.9471	Not Significant
21	Gonadal segmental translucence	F	1.0000	Not Significant
		M	0.0882	Not Significant
22	Gonadal pearling	F	1.0000	Not Significant
		M	1.0000	Not Significant
23	Melanophores decreased in ovaries	F	0.8374	Not Significant
24	Internal melanophores in testes	M	1.0000	Not Significant
25	Frequency of males among just males and females	-	0.2431	Not Significant
26	Frequency of males	-	0.2286	Not Significant
28	Frequency of intersex	-	1.0000	Not Significant
29	Frequency of mixed sex	-	0.8960	Not Significant

TABLE 2C.2. IGB GROSS -Testing All Measurement-Based Tank Endpoints for **Normality and Homogeneity of Variances**

Index #	Characteristic	Sex	Analysis Group	p-value	S or NS	p-value	S or NS
3	Age at completion of metamorphosis	F	ATZ	0.5724	NS	0.3614	NS
			E2	0.7569	NS	0.7897	NS
		M	ATZ	0.1161	NS	0.7848	NS
			E2	0.2184	NS	0.6242	NS
4	Body weight	F	ATZ	0.5089	NS	0.1795	NS
			E2	0.3249	NS	0.6247	NS
		M	ATZ	0.3149	NS	0.6896	NS
			E2	0.7570	NS	0.4012	NS
5	Snout-to-vent length	F	ATZ	0.5385	NS	0.1471	NS
			E2	0.8348	NS	0.9839	NS
		M	ATZ	0.9374	NS	0.2055	NS
			E2	0.7065	NS	0.0508	NS
6	Gonadal image area	F	ATZ	0.8538	NS	0.1690	NS
			E2	0.7382	NS	0.8300	NS
		M	ATZ	0.7813	NS	0.9692	NS
			E2	0.0085	S	0.0177	NS

TABLE 2C.3. IGB GROSS -Testing Transformations of Mean Gonadal Image Area in Females for Normality and Homogeneity of Variances

Index #	Characteristic	Sex	Analysis Group	Power in Box-Cox Transformation	Shapiro-Wilk Test for Non-Normality: p-value	Levene's Test for Heterogeneity of Variances: p-value	Acceptability
6	Gonadal image area	M	E2	1.0	0.0085	0.0177	
				0.5	0.0095	0.0258	
				0.0	0.0072	0.0385	
				-0.5	0.0048	0.0478	
				-1.0	0.0025	0.0596	

TABLE 2C.4. IGB GROSS **-E2 Comparison to Control**: Measurement-Based Tank Measures and Incidence-Based Measures Not Grouped by Sex

Index #	Characteristic	Test	Test Type	Sex	p-value	S or NS
1	Tadpole mortality	Wilcoxon-Mann-Whitney	1+	-	0.3453	NS
2	Failure to complete metamorphosis	Wilcoxon-Mann-Whitney	1+	-	1.0000	NS
3	Age at completion of metamorphosis	t-test	2	F	0.0428	S
		t-test		M	0.0001	S
4	Body weight	t-test	2	F	0.6892	NS
		t-test		M	0.1674	NS
5	Snout-to-vent length	t-test	2	F	0.2964	NS
		t-test		M	0.3175	NS
6	Gonadal image area	t-test	2	F	0.7523	NS
		Wilcoxon-Mann-Whitney		M	0.0071	S
7	Intersex (gross observation)	Wilcoxon-Mann-Whitney	1+	-	1.0000	NS
8	Mixed tissue types (gross observation)	Wilcoxon-Mann-Whitney	1+	-	0.0013	S
25	Frequency of males among just males and females	Wilcoxon-Mann-Whitney	1-	-	0.0006	S
26	Frequency of males	Wilcoxon-Mann-Whitney	1-	-	<0.0001	S
28	Frequency of intersex	Wilcoxon-Mann-Whitney	1+	-	1.0000	NS
29	Frequency of mixed sex	Wilcoxon-Mann-Whitney	1+	-	<0.0001	S

TABLE 2C.5. IGB GROSS -Test for **Tank Effects among E2 Males and Control Males** for Incidence-Based Measures Grouped by Sex: Chi-Square Test and Tippet's minimum p-value

Index #	Characteristic	p-value	Significant or Not Significant
9	Gonadal adhesions	1.0000	Not Significant
10	Gonadal aplasia	1.0000	Not Significant
11	Gonadal segmental aplasia	0.4387	Not Significant
12	Gonadal bifurcation	1.0000	Not Significant
13	Gonadal deformity	0.7868	Not Significant
14	Gonadal displacement	0.9562	Not Significant
15	Fused gonads	1.0000	Not Significant
16	Gonadal hypertrophy	0.0879	Not Significant
17	Gonadal segmental hypertrophy	1.0000	Not Significant
18	Gonadal hypoplasia	0.5456	Not Significant
19	Gonadal segmental hypoplasia	0.5124	Not Significant
20	Gonadal translucence	0.0657	Not Significant
21	Gonadal segmental translucence	0.2540	Not Significant
22	Gonadal pearling	1.0000	Not Significant
24	Internal melanophores in testes	1.0000	Not Significant

TABLE 2C.6. IGB GROSS -**Comparison of E2 Males to Control Males** for Incidence-Based Measures Grouped by Sex: Fisher's Exact Test

Index #	Characteristic	Test Type	p-value	Significant or Not Significant
9	Gonadal adhesions	1+	1.0000	Not Significant
10	Gonadal aplasia	1+	1.0000	Not Significant
11	Gonadal segmental aplasia	1+	<0.0001	Significant
12	Gonadal bifurcation	1+	1.0000	Not Significant
13	Gonadal deformity	1+	1.0000	Not Significant
14	Gonadal displacement	1+	0.4674	Not Significant
15	Fused gonads	1+	1.0000	Not Significant
16	Gonadal hypertrophy	1+	0.3206	Not Significant
17	Gonadal segmental hypertrophy	1+	1.0000	Not Significant
18	Gonadal hypoplasia	1+	0.7451	Not Significant
19	Gonadal segmental hypoplasia	1+	1.0000	Not Significant
20	Gonadal translucence	1+	<0.0001	Significant
21	Gonadal segmental translucence	1+	0.2381	Not Significant
22	Gonadal pearling	1+	1.0000	Not Significant
24	Internal melanophores in testes	1+	1.0000	Not Significant

TABLE 2C.7. IGB GROSS -**Comparison of E2 Females to Control Females** for Incidence-Based Measures Grouped by Sex: Wilcoxon-Mann-Whitney Test

Index #	Characteristic	Test Type	p-value	Significant or Not Significant
9	Gonadal adhesions	1+	1.0000	Not Significant
10	Gonadal aplasia	1+	1.0000	Not Significant
11	Gonadal segmental aplasia	1+	0.3333	Not Significant
12	Gonadal bifurcation	1+	1.0000	Not Significant
13	Gonadal deformity	1+	0.9438	Not Significant
14	Gonadal displacement	1+	0.7650	Not Significant
15	Fused gonads	1+	0.7233	Not Significant
16	Gonadal hypertrophy	1+	0.8782	Not Significant
17	Gonadal segmental hypertrophy	1+	1.0000	Not Significant
18	Gonadal hypoplasia	1+	0.0032	Significant
19	Gonadal segmental hypoplasia	1+	0.0277	Significant
20	Gonadal translucence	1+	0.7233	Not Significant
21	Gonadal segmental translucence	1+	0.5652	Not Significant
22	Gonadal pearling	1+	1.0000	Not Significant
23	Melanophores decreased in ovaries	1+	0.3295	Not Significant

TABLE 2C.8. IGB GROSS -**Atrazine Treatment Groups and Controls**: Measurement-Based Tank Measures and Incidence-Based Measures Not Grouped by Sex

Index #	Characteristic	Test	Test Type	Sex	p-value	Treatment-Control Differences
1	Tadpole mortality	Kruskal-Wallis	1+	-	0.9908	None Significant
2	Failure to complete metamorphosis	Kruskal-Wallis	1+	-	0.7234	None Significant
3	Age at completion of metamorphosis	One-Way ANOVA F-Test	2	F	0.3234	None Significant
				M	0.8856	None Significant
4	Body weight	One-Way ANOVA F-Test	2	F	0.0187	Separate Testing Required
				M	0.6685	None Significant
5	Snout-to-vent length	One-Way ANOVA F-Test	2	F	0.0146	Separate Testing Required
				M	0.3054	None Significant
6	Gonadal image area	One-Way ANOVA F-Test	2	F	0.1591	None Significant
				M	0.0753	None Significant
7	Intersex (gross observation)	Kruskal-Wallis	1+	-	1.0000	None Significant
8	Mixed tissue types (gross observation)	Kruskal-Wallis	1+	-	1.0000	None Significant
25	Frequency of males among just males and females	Kruskal-Wallis	1-	-	0.7709	None Significant
26	Frequency of males	Kruskal-Wallis	1-	-	0.7732	None Significant
28	Frequency of intersex	Kruskal-Wallis	1+	-	1.0000	None Significant
29	Frequency of mixed sex	Kruskal-Wallis	1+	-	0.7160	None Significant

TABLE 2C.9. IGB GROSS -Pairwise ANOVA contrast comparisons of the **body weight** in the five atrazine female treatment groups with control **females**

Atrazine Dose (ppm)	body weight	Test Type	p-value	Significant or Not Significant
0.0 (Controls)	0.555	-	-	-
0.01	0.516	2	0.0113	Significant
0.10	0.543	2	0.4190	Not Significant
1.00	0.518	2	0.0154	Significant
25.00	0.555	2	0.9968	Not Significant
100.00	0.517	2	0.0138	Significant

TABLE 2C.10 IGB GROSS -Pairwise ANOVA contrast comparisons of the **snout-to-vent length** in the five atrazine female treatment groups with control **females**

Atrazine Dose (ppm)	snout-to-vent length	Test Type	p-value	Significant or Not Significant
0.0 (Controls)	18.351	-	-	-
0.01	17.904	2	0.0091	Significant
0.10	18.207	2	0.3865	Not Significant
1.00	17.959	2	0.0212	Significant
25.00	18.284	2	0.6834	Not Significant
100.00	17.848	2	0.0036	Significant

TABLE 2C.11 IGB GROSS -**Atrazine Treatment Groups and Controls**: Incidence-Based Measures Grouped by Sex: Kruskal-Wallis Test

Index #	Characteristic	Test Type	Sex	p-value	Treatment-Control Differences
9	Gonadal adhesions	1+	F	1.0000	None Significant
			M	1.0000	None Significant
10	Gonadal aplasia	1+	F	1.0000	None Significant
			M	1.0000	None Significant
11	Gonadal segmental aplasia	1+	F	0.7146	None Significant
			M	0.7221	None Significant
12	Gonadal bifurcation	1+	F	1.0000	None Significant
			M	1.0000	None Significant
13	Gonadal deformity	1+	F	0.8952	None Significant
			M	1.0000	None Significant
14	Gonadal displacement	1+	F	0.4563	None Significant
			M	0.8240	None Significant
15	Fused gonads	1+	F	0.6857	None Significant
			M	0.0683	Separate Testing Required
16	Gonadal hypertrophy	1+	F	0.7191	None Significant
			M	0.0484	Separate Testing Required
17	Gonadal segmental hypertrophy	1+	F	0.0912	Separate Testing Required
			M	0.5029	None Significant
18	Gonadal hypoplasia	1+	F	0.1593	None Significant
			M	0.8604	None Significant
19	Gonadal segmental hypoplasia	1+	F	0.0947	Separate Testing Required
			M	0.3328	None Significant
20	Gonadal translucence	1+	F	0.3802	None Significant
			M	0.4317	None Significant
21	Gonadal segmental translucence	1+	F	1.0000	None Significant
			M	0.4204	None Significant
22	Gonadal pearling	1+	F	1.0000	None Significant
			M	1.0000	None Significant
23	Melanophores decreased in ovaries	1+	F	0.2437	None Significant
24	Internal melanophores in testes	1+	M	0.7136	None Significant

TABLE 2C.12. IGB GROSS -Wilcoxon-Mann-Whitney comparisons of the percent with **fused gonads** in the five atrazine male treatment groups with control **males**

Atrazine Dose (ppm)	Mean Rank (Treatment) - Mean Rank (Control)	Test Type	p-value	Significant or Not Significant
0.01	1.500	1+	0.3333	Not Significant
0.10	0.000	1+	1.0000	Not Significant
1.00	0.000	1+	1.0000	Not Significant
25.00	0.000	1+	1.0000	Not Significant
100.00	3.000	1+	0.1014	Not Significant

TABLE 2C.13. IGB GROSS -Wilcoxon-Mann-Whitney comparisons of the percent with **gonadal hypertrophy** in the five atrazine male treatment groups with control **males**

Atrazine Dose (ppm)	Mean Rank (Treatment) - Mean Rank (Control)	Test Type	p-value	Significant or Not Significant
0.01	2.531	1+	0.2125	Not Significant
0.10	-5.438	1+	0.9641	Not Significant
1.00	-2.250	1+	0.7695	Not Significant
25.00	-3.844	1+	0.8964	Not Significant
100.00	-6.844	1+	0.9891	Not Significant

TABLE 2C.14. IGB GROSS -Wilcoxon-Mann-Whitney comparisons of the percent with **gonadal segmental hypertrophy** in the five atrazine female treatment groups with control **females**

Atrazine Dose (ppm)	Mean Rank (Treatment) - Mean Rank (Control)	Test Type	p-value	Significant or Not Significant
0.01	1.688	1+	0.1963	Not Significant
0.10	-1.500	1+	1.0000	Not Significant
1.00	0.188	1+	0.4071	Not Significant
25.00	5.156	1+	0.0152	Significant
100.00	0.000	1+	0.5652	Not Significant

TABLE 2C.15. IGB GROSS -Wilcoxon-Mann-Whitney comparisons of the percent with **gonadal segmental hypoplasia** in the five atrazine female treatment groups with control **females**

Atrazine Dose (ppm)	Mean Rank (Treatment) - Mean Rank (Control)	Test Type	p-value	Significant or Not Significant
0.01	0.375	1+	0.4700	Not Significant
0.10	5.813	1+	0.0213	Significant
1.00	0.281	1+	0.4879	Not Significant
25.00	5.906	1+	0.0198	Significant
100.00	2.813	1+	0.1550	Not Significant

TABLE 2C.16. IGB GROSS – Atrazine Treatment Groups and Controls: **Trend Tests**

Index #	Characteristic	# of Atrazine Doses	High Dose (ppm)	Test Type	Sex	p-value	Significant or Not Significant
1	Tadpole mortality	5	100	1+	-	0.4587	Not Significant
2	Failure to complete metamorphosis	5	100	1+	-	0.2799	Not Significant
3	Age at completion of metamorphosis	5	100	2	F	0.9520	Not Significant
		5	100		M	0.9906	Not Significant
4	Body weight	5	100	2	F	0.1250	Not Significant
		5	100		M	0.1543	Not Significant
5	Snout-to-vent length	5	100	2	F	0.0868	Not Significant
		5	100		M	0.0636	Not Significant
6	Gonadal image area	5	100	2	F	0.2382	Not Significant
		5	100		M	0.0195	Significant
		4	25			0.0462	Significant
		3	1			0.0210	Significant
		2	0.1			0.0609	Not Significant
7	Intersex (gross observation)	5	100	1+	-	1.0000	Not Significant
8	Mixed tissue types (gross observation)	5	100	1+	-	1.0000	Not Significant
9	Gonadal adhesions	5	100	1+	F	1.0000	Not Significant
		5	100		M	1.0000	Not Significant
10	Gonadal aplasia	5	100	1+	F	1.0000	Not Significant
		5	100		M	1.0000	Not Significant
11	Gonadal segmental aplasia	5	100	1+	F	0.1437	Not Significant
		5	100		M	0.1918	Not Significant
12	Gonadal bifurcation	5	100	1+	F	1.0000	Not Significant
		5	100		M	1.0000	Not Significant
13	Gonadal deformity	5	100	1+	F	>0.6716	Not Significant
		5	100		M	>0.7111	Not Significant
14	Gonadal displacement	5	100	1+	F	>0.6464	Not Significant
		5	100		M	0.2840	Not Significant
15	Fused gonads	5	100	1+	F	0.2328	Not Significant
		5	100		M	0.0664	Not Significant
16	Gonadal hypertrophy	5	100	1+	F	>0.6887	Not Significant
		5	100		M	>0.9949	Not Significant
17	Gonadal segmental hypertrophy	5	100	1+	F	0.1720	Not Significant
		5	100		M	0.2394	Not Significant

TABLE 2C.16. IGB GROSS – Atrazine Treatment Groups and Controls: **Trend Tests**

Index #	Characteristic	# of Atrazine Doses	High Dose (ppm)	Test Type	Sex	p-value	Significant or Not Significant
18	Gonadal hypoplasia	5	100	1+	F	>0.9169	Not Significant
		5	100		M	0.3887	Not Significant
19	Gonadal segmental hypoplasia	5	100	1+	F	0.0370	Significant
		4	25			0.0259	Significant
		3	1			0.1951	Not Significant
		5	100		M	>0.5814	Not Significant
20	Gonadal translucence	5	100	1+	F	>0.9550	Not Significant
		5	100		M	0.1349	Not Significant
21	Gonadal segmental translucence	5	100	1+	F	>0.5873	Not Significant
		5	100		M	>0.7887	Not Significant
22	Gonadal pearling	5	100	1+	F	1.0000	Not Significant
		5	100		M	1.0000	Not Significant
23	Melanophores decreased in ovaries	5	100	1+	F	0.0125	Significant
		4	25			0.0592	Not Significant
24	Internal melanophores in testes	5	100	1+	M	0.2896	Not Significant
25	Frequency of males among just males and females	5	100	1-	-	>0.8677	Not Significant
26	Frequency of males	5	100	1-	-	>0.8598	Not Significant
28	Frequency of intersex	5	100	1+	-	1.0000	Not Significant
29	Frequency of mixed sex	5	100	1+	-	0.2806	Not Significant

TABLE 2C.17. IGB EPL – Tests for **Tank Cluster Effects:** Wilcoxon-Mann-Whitney Tests and Tippett's Minimum p-value Method

Index #	Characteristic	Sex	p-value	Significant or Not Significant
1	Segmental hypoplasia (Grade>0)	F	0.9612	Not Significant
		M	0.1837	Not Significant
	Segmental hypoplasia (Grade>1)	F	1.0000	Not Significant
		M	0.9998	Not Significant
2	Segmental aplasia (Grade>0)	F	1.0000	Not Significant
		M	0.1029	Not Significant
	Segmental aplasia (Grade>1)	F	1.0000	Not Significant
		M	1.0000	Not Significant
3	Decreased germ cells in gonad (Grade>0)	F	0.1837	Not Significant
		M	0.9471	Not Significant
	Decreased germ cells in gonad (Grade>1)	F	0.5724	Not Significant
		M	0.4660	Not Significant
4	Dilated testis tubules (Grade>0)	M	<0.0001	Significant
	Dilated testis tubules (Grade>1)	M	0.1479	Not Significant
5	Dividing gonocytes in testes (Grade>0)	M	0.5254	Not Significant
	Dividing gonocytes in testes (Grade>1)	M	0.7979	Not Significant
6	Internal melanophores in testis (Grade>0)	M	0.3376	Not Significant
	Internal melanophores in testis (Grade>1)	M	0.1837	Not Significant
7	Decreased ovarian pigmentation (Grade>0)	F	0.9287	Not Significant
	Decreased ovarian pigmentation (Grade>1)	F	0.9334	Not Significant
8	Decreased ovarian cavity size (Grade>0)	F	0.7319	Not Significant
	Decreased ovarian cavity size (Grade>1)	F	0.9652	Not Significant
9	Increased ovarian cavity size (Grade>0)	F	0.9998	Not Significant
	Increased ovarian cavity size (Grade>1)	F	0.9652	Not Significant
10	Ovarian interstitial fibrosis (Grade>0)	F	0.3376	Not Significant
	Ovarian interstitial fibrosis (Grade>1)	F	1.0000	Not Significant
11	Mononuclear cell infiltrates in ovary (Grade>0)	F	0.5724	Not Significant
	Mononuclear cell infiltrates in ovary (Grade>1)	F	0.8752	Not Significant
12	One or more immature ovaries	F	0.9711	Not Significant
	One or more unable to stage ovaries	F	0.2605	Not Significant
13	Fused kidneys (Grade>0)	F	0.4660	Not Significant
		M	0.4660	Not Significant
	Fused kidneys (Grade>1)	F	1.0000	Not Significant
		M	0.9801	Not Significant

TABLE 2C.17. IGB EPL – Tests for **Tank Cluster Effects:** Wilcoxon-Mann-Whitney Tests and Tippett's Minimum p-value Method

Index #	Characteristic	Sex	p-value	Significant or Not Significant
14	Mineralization (Grade>0)	F	0.7319	Not Significant
		M	0.1837	Not Significant
	Mineralization (Grade>1)	F	0.9801	Not Significant
		M	1.0000	Not Significant
15	Other renal diagnoses (Grade>0)	F	1.0000	Not Significant
		M	1.0000	Not Significant
	Other renal diagnoses (Grade>1)	F	1.0000	Not Significant
		M	1.0000	Not Significant

TABLE 2C.18. IGB EPL-Test for **Tank Effects among E2 Males** and Control Males for Incidence-Based Measures Grouped by Sex: Chi-Square Test and Tippet's minimum p-value

Index #	Characteristic	p-value	Significant or Not Significant
1	Segmental hypoplasia (Grade>0)	0.4549	Not Significant
	Segmental hypoplasia (Grade>1)	0.9158	Not Significant
2	Segmental aplasia (Grade>0)	0.4435	Not Significant
	Segmental aplasia (Grade>1)	0.7824	Not Significant
3	Decreased germ cells in gonad (Grade>0)	0.9167	Not Significant
	Decreased germ cells in gonad (Grade>1)	0.9180	Not Significant
4	Dilated testis tubules (Grade>0)	0.0645	Not Significant
	Dilated testis tubules (Grade>1)	0.8200	Not Significant
5	Dividing gonocytes in testes (Grade>0)	0.1570	Not Significant
	Dividing gonocytes in testes (Grade>1)	0.2043	Not Significant
6	Internal melanophores in testis (Grade>0)	0.3128	Not Significant
	Internal melanophores in testis (Grade>1)	0.3767	Not Significant
13	Fused kidneys (Grade>0)	0.0741	Not Significant
	Fused kidneys (Grade>1)	0.7755	Not Significant
14	Mineralization (Grade>0)	0.2170	Not Significant
	Mineralization (Grade>1)	1.0000	Not Significant
15	Other renal diagnoses (Grade>0)	0.9983	Not Significant
	Other renal diagnoses (Grade>1)	1.0000	Not Significant

TABLE 2C.19. IGB EPL- **Comparison of E2 Males to Control Males** for Incidence-Based Measures Grouped by Sex: Fisher's Exact Test

Index #	Characteristic	Test Type	p-value	Significant or Not Significant
1	Segmental hypoplasia (Grade>0)	1+	0.0030	Significant
	Segmental hypoplasia (Grade>1)	1+	0.0129	Significant
2	Segmental aplasia (Grade>0)	1+	0.7768	Not Significant
	Segmental aplasia (Grade>1)	1+	1.0000	Not Significant
3	Decreased germ cells in gonad (Grade>0)	1+	0.3786	Not Significant
	Decreased germ cells in gonad (Grade>1)	1+	0.0707	Not Significant
4	Dilated testis tubules (Grade>0)	1+	<0.0001	Significant
	Dilated testis tubules (Grade>1)	1+	<0.0001	Significant
5	Dividing gonocytes in testes (Grade>0)	1+	<0.0001	Significant
	Dividing gonocytes in testes (Grade>1)	1+	0.0007	Significant
6	Internal melanophores in testis (Grade>0)	1+	0.0428	Significant
	Internal melanophores in testis (Grade>1)	1+	0.0003	Significant
13	Fused kidneys (Grade>0)	1+	0.1973	Not Significant
	Fused kidneys (Grade>1)	1+	1.0000	Not Significant
14	Mineralization (Grade>0)	2	0.3433	Not Significant
	Mineralization (Grade>1)	2	1.0000	Not Significant
15	Other renal diagnoses (Grade>0)	2	1.0000	Not Significant
	Other renal diagnoses (Grade>1)	2	1.0000	Not Significant

TABLE 2C.20. IGB EPL- **Comparison of E2 Females to Control Females** for Incidence-Based Measures Grouped by Sex: Wilcoxon-Mann-Whitney Test

Index #	Characteristic	Test Type	p-value	Significant or Not Significant
1	Segmental hypoplasia (Grade>0)	1+	0.0117	Significant
	Segmental hypoplasia (Grade>1)	1+	1.0000	Not Significant
2	Segmental aplasia (Grade>0)	1+	0.5652	Not Significant
	Segmental aplasia (Grade>1)	1+	0.5652	Not Significant
3	Decreased germ cells in gonad (Grade>0)	1+	0.5896	Not Significant
	Decreased germ cells in gonad (Grade>1)	1+	0.2219	Not Significant
7	Decreased ovarian pigmentation (Grade>0)	1+	0.7227	Not Significant
	Decreased ovarian pigmentation (Grade>1)	1+	0.7900	Not Significant
8	Decreased ovarian cavity size (Grade>0)	1+	0.2002	Not Significant
	Decreased ovarian cavity size (Grade>1)	1+	0.1014	Not Significant
9	Increased ovarian cavity size (Grade>0)	1+	0.0001	Significant
	Increased ovarian cavity size (Grade>1)	1+	0.1014	Not Significant
10	Ovarian interstitial fibrosis (Grade>0)	1+	0.2757	Not Significant
	Ovarian interstitial fibrosis (Grade>1)	1+	0.0698	Not Significant
11	Mononuclear cell infiltrates in ovary (Grade>0)	1+	0.3763	Not Significant
	Mononuclear cell infiltrates in ovary (Grade>1)	1+	0.3123	Not Significant
12	One or more immature ovaries	1+	0.7818	Not Significant
	One or more unable to stage ovaries	2	0.4598	Not Significant
13	Fused kidneys (Grade>0)	1+	0.0508	Not Significant
	Fused kidneys (Grade>1)	1+	0.3333	Not Significant
14	Mineralization (Grade>0)	2	0.0669	Not Significant
	Mineralization (Grade>1)	2	1.0000	Not Significant
15	Other renal diagnoses (Grade>0)	2	1.0000	Not Significant
	Other renal diagnoses (Grade>1)	2	0.3333	Not Significant

TABLE 2C.21. IGB EPL- **Atrazine Treatment Groups and Controls**: Incidence-Based Measures Grouped by Sex: Kruskal-Wallis Test

Index #	Characteristic	Test Type	Sex	p-value	Treatment-Control Differences
1	Segmental hypoplasia (Grade>0)	1+	F	0.6903	None Significant
			M	0.4057	None Significant
	Segmental hypoplasia (Grade>1)	1+	F	0.7892	None Significant
			M	0.8495	None Significant
2	Segmental aplasia (Grade>0)	1+	F	0.7917	None Significant
			M	0.1260	None Significant
	Segmental aplasia (Grade>1)	1+	F	1.0000	None Significant
			M	0.1762	None Significant
3	Decreased germ cells in gonad (Grade>0)	1+	F	0.3651	None Significant
			M	0.9994	None Significant
	Decreased germ cells in gonad (Grade>1)	1+	F	0.6066	None Significant
			M	0.5467	None Significant
4	Dilated testis tubules (Grade>0)	1+	M	0.3586	None Significant
	Dilated testis tubules (Grade>1)	1+	M	0.9228	None Significant
5	Dividing gonocytes in testes (Grade>0)	1+	M	0.3109	None Significant
	Dividing gonocytes in testes (Grade>1)	1+	M	0.2301	None Significant
6	Internal melanophores in testis (Grade>0)	1+	M	0.3460	None Significant
	Internal melanophores in testis (Grade>1)	1+	M	0.0168	Separate Testing Required
7	Decreased ovarian pigmentation (Grade>0)	1+	F	0.2996	None Significant
	Decreased ovarian pigmentation (Grade>1)	1+	F	0.1134	None Significant
8	Decreased ovarian cavity size (Grade>0)	1+	F	0.1350	None Significant
	Decreased ovarian cavity size (Grade>1)	1+	F	0.1218	None Significant
9	Increased ovarian cavity size (Grade>0)	1+	F	0.3421	None Significant
	Increased ovarian cavity size (Grade>1)	1+	F	0.7112	None Significant
10	Ovarian interstitial fibrosis (Grade>0)	1+	F	0.2035	None Significant
	Ovarian interstitial fibrosis (Grade>1)	1+	F	0.8307	None Significant
11	Mononuclear cell infiltrates in ovary (Grade>0)	1+	F	0.1435	None Significant
	Mononuclear cell infiltrates in ovary (Grade>1)	1+	F	0.3196	None Significant
12	One or more immature ovaries	1+	F	0.6671	None Significant
	One or more unable to stage ovaries	2	F	0.4053	None Significant
13	Fused kidneys (Grade>0)	1+	F	0.9783	None Significant
			M	0.2772	None Significant
	Fused kidneys (Grade>1)	1+	F	0.5610	None Significant
			M	0.8900	None Significant
14	Mineralization (Grade>0)	2	F	0.0990	None Significant
			M	0.0882	None Significant

TABLE 2C.21. IGB EPL- **Atrazine Treatment Groups and Controls**: Incidence-Based Measures Grouped by Sex: Kruskal-Wallis Test

Index #	Characteristic	Test Type	Sex	p-value	Treatment-Control Differences
	Mineralization (Grade>1)	2	F	0.2424	None Significant
			M	0.7124	None Significant
15	Other renal diagnoses (Grade>0)	2	F	0.1714	None Significant
			M	0.6064	None Significant
	Other renal diagnoses (Grade>1)	2	F	0.5074	None Significant
			M	0.5073	None Significant

TABLE 2C.22. IGB EPL- Percent of Males with **Internal melanophores in testis (Grade>1)**: Wilcoxon-Mann-Whitney Comparisons of the Five Atrazine Treatment Groups with Controls

Atrazine Dose (ppm)	Mean Rank (Treatment) - Mean Rank (Control)	Test Type	p-value	Significant or Not Significant
0.01	2.906	1+	0.1778	Not Significant
0.10	1.875	1+	0.2770	Not Significant
1.00	-1.594	1+	0.7016	Not Significant
25.00	-3.094	1+	0.8491	Not Significant
100.00	-5.250	1+	0.9638	Not Significant

Index #	Characteristic	# of Atrazine Doses	High Dose (ppm)	Test Type	Sex	p-value	Significant or Not Significant
1	Segmental hypoplasia (Grade>0)	5	100	1+	F	>0.8256	Not Significant
		5	100		M	0.2849	Not Significant
	Segmental hypoplasia (Grade>1)	5	100	1+	F	0.4255	Not Significant
		5	100		M	>0.5745	Not Significant
2	Segmental aplasia (Grade>0)	5	100	1+	F	>0.5291	Not Significant
		5	100		M	0.0239	Significant
		4	25			0.0401	Significant
		3	1			>0.5354	Not Significant
	Segmental aplasia (Grade>1)	5	100	1+	F	>0.7246	Not Significant
		5	100		M	0.0280	Significant
		4	25			0.2718	Not Significant
3	Decreased germ cells in gonad (Grade>0)	5	100	1+	F	0.3703	Not Significant
		5	100		M	0.4812	Not Significant
	Decreased germ cells in gonad (Grade>1)	5	100	1+	F	0.4459	Not Significant
		5	100		M	>0.8389	Not Significant
4	Dilated testis tubules (Grade>0)	5	100	1+	M	0.1099	Not Significant
	Dilated testis tubules (Grade>1)	5	100	1+	M	0.1874	Not Significant
5	Dividing gonocytes in testes (Grade>0)	5	100	1+	M	0.2958	Not Significant
	Dividing gonocytes in testes (Grade>1)	5	100	1+	M	0.2826	Not Significant
6	Internal melanophores in testis (Grade>0)	5	100	1+	M	>0.8238	Not Significant
	Internal melanophores in testis (Grade>1)	5	100	1+	M	>0.9967	Not Significant
7	Decreased ovarian pigmentation (Grade>0)	5	100	1+	F	0.0194	Significant
		4	25			0.1193	Not Significant
	Decreased ovarian pigmentation (Grade>1)	5	100	1+	F	0.0028	Significant
		4	25			0.0158	Significant
		3	1			0.0195	Significant
		2	0.1			0.2039	Not Significant
8	Decreased ovarian cavity size (Grade>0)	5	100	1+	F	0.0201	Significant
		4	25			0.0028	Significant
		3	1			0.0414	Significant
		2	0.1			0.1555	Not Significant

Index #	Characteristic	# of Atrazine Doses	High Dose (ppm)	Test Type	Sex	p-value	Significant or Not Significant
	Decreased ovarian cavity size (Grade>1)	5	100	1+	F	0.3071	Not Significant
9	Increased ovarian cavity size (Grade>0)	5	100	1+	F	0.0871	Not Significant
	Increased ovarian cavity size (Grade>1)	5	100	1+	F	>0.5690	Not Significant
10	Ovarian interstitial fibrosis (Grade>0)	5	100	1+	F	>0.8817	Not Significant
	Ovarian interstitial fibrosis (Grade>1)	5	100	1+	F	>0.6466	Not Significant
11	Mononuclear cell infiltrates in ovary (Grade>0)	5	100	1+	F	0.4357	Not Significant
	Mononuclear cell infiltrates in ovary (Grade>1)	5	100	1+	F	>0.7286	Not Significant
12	One or more immature ovaries	5	100	1+	F	>0.8914	Not Significant
	One or more unable to stage ovaries	5	100	2	F	0.1662	Not Significant
13	Fused kidneys (Grade>0)	5	100	1+	F	0.3970	Not Significant
		5	100		M	0.0138	Significant
		4	25			0.1431	Not Significant
	Fused kidneys (Grade>1)	5	100	1+	F	0.3045	Not Significant
		5	100		M	0.3729	Not Significant
14	Mineralization (Grade>0)	5	100	2	F	0.2978	Not Significant
		5	100		M	0.0429	Significant
		4	25			0.0529	Not Significant
	Mineralization (Grade>1)	5	100	2	F	0.4759	Not Significant
		5	100		M	0.5721	Not Significant
15	Other renal diagnoses (Grade>0)	5	100	2	F	0.9713	Not Significant
		5	100		M	0.1418	Not Significant
	Other renal diagnoses (Grade>1)	5	100	2	F	0.7142	Not Significant
		5	100		M	0.7145	Not Significant

APPENDIX 2D
BASIC SUMMARY STATISTICS WIL AND IGB GROSS MORPHOLOGY AND HISTOPATHOLOGY

TABLE 2D.1. WILDLIFE GROSS - Basic Summary Statistics for all Response Variables

No.	Response Variable (all except #s 3,4,5,6 are expressed as percents)	Sex	Chemical	Dose (ug/L)	No. of Tanks	Mean	Median	Min	Max	Standard Deviation	Standard Error
1	Tadpole mortality	None	E2	0.20	8	2.500	2.000	0.000	8.000	2.976	1.052
			Ctrl	0.00	8	6.500	4.000	0.000	20.000	6.740	2.383
			ATZ	0.01	8	1.500	0.000	0.000	4.000	2.070	0.732
			ATZ	0.10	8	5.000	6.000	0.000	12.000	4.660	1.648
			ATZ	1.00	7	4.571	4.000	0.000	12.000	4.860	1.837
			ATZ	25.00	8	6.000	4.000	4.000	12.000	3.024	1.069
			ATZ	100.00	8	3.542	4.000	0.000	8.333	3.404	1.203
2	Failure to complete metamorphosis	None	E2	0.20	8	0.000	0.000	0.000	0.000	0.000	0.000
			Ctrl	0.00	8	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	0.01	8	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	0.10	8	0.500	0.000	0.000	4.000	1.414	0.500
			ATZ	1.00	7	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	25.00	8	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	100.00	8	0.521	0.000	0.000	4.167	1.473	0.521
3	Age at completion of metamorphosis	F	E2	0.20	8	54.001	53.895	52.313	56.286	1.391	0.492
			Ctrl	0.00	8	52.925	52.800	51.500	55.000	1.099	0.389
			ATZ	0.01	8	51.564	51.515	48.889	53.294	1.448	0.512
			ATZ	0.10	8	51.984	52.554	50.333	53.417	1.198	0.424
			ATZ	1.00	7	51.915	51.545	49.750	53.722	1.393	0.526
			ATZ	25.00	8	52.715	52.679	51.400	54.786	1.026	0.363
			ATZ	100.00	8	52.900	52.829	51.125	55.462	1.550	0.548
		M	E2	0.20	8	55.153	55.792	51.333	58.222	2.330	0.824
			Ctrl	0.00	8	52.217	52.343	49.667	55.111	1.758	0.622
			ATZ	0.01	8	51.600	51.513	50.385	53.375	0.939	0.332

TABLE 2D.1. WILDLIFE GROSS - Basic Summary Statistics for all Response Variables

No.	Response Variable (all except #s 3,4,5,6 are expressed as percents)	Sex	Chemical	Dose (ug/L)	No. of Tanks	Mean	Median	Min	Max	Standard Deviation	Standard Error
			ATZ	0.10	8	52.573	52.792	49.400	56.100	2.236	0.790
			ATZ	1.00	7	52.869	52.273	50.091	56.000	2.374	0.897
			ATZ	25.00	8	51.392	51.525	50.000	52.727	0.883	0.312
			ATZ	100.00	8	52.101	52.048	49.727	54.500	1.581	0.559
4	Body weight	F	E2	0.20	8	0.617	0.617	0.585	0.643	0.018	0.006
			Ctrl	0.00	8	0.609	0.606	0.563	0.676	0.041	0.014
			ATZ	0.01	8	0.581	0.588	0.503	0.652	0.047	0.016
			ATZ	0.10	8	0.566	0.576	0.496	0.633	0.044	0.016
			ATZ	1.00	7	0.579	0.565	0.526	0.669	0.050	0.019
			ATZ	25.00	8	0.597	0.594	0.536	0.655	0.038	0.013
			ATZ	100.00	8	0.575	0.586	0.478	0.619	0.047	0.017
		M	E2	0.20	8	0.585	0.579	0.527	0.667	0.051	0.018
			Ctrl	0.00	8	0.554	0.546	0.522	0.629	0.034	0.012
			ATZ	0.01	8	0.580	0.587	0.498	0.640	0.043	0.015
			ATZ	0.10	8	0.559	0.549	0.499	0.654	0.047	0.017
			ATZ	1.00	7	0.558	0.546	0.483	0.651	0.054	0.020
			ATZ	25.00	8	0.572	0.570	0.525	0.641	0.040	0.014
			ATZ	100.00	8	0.581	0.578	0.496	0.668	0.054	0.019
5	Snout-to-vent length	F	E2	0.20	8	18.794	18.737	18.500	19.214	0.242	0.085
			Ctrl	0.00	8	18.747	18.722	18.273	19.375	0.414	0.146
			ATZ	0.01	8	18.543	18.500	18.111	19.222	0.321	0.114
			ATZ	0.10	8	18.395	18.519	17.455	19.083	0.479	0.169
			ATZ	1.00	7	18.426	18.400	17.750	19.273	0.549	0.208
			ATZ	25.00	8	18.645	18.679	17.909	19.000	0.334	0.118
			ATZ	100.00	8	18.397	18.624	17.250	18.778	0.519	0.184
		M	E2	0.20	8	18.615	18.875	17.833	19.333	0.579	0.205

TABLE 2D.1. WILDLIFE GROSS - Basic Summary Statistics for all Response Variables

No.	Response Variable (all except #s 3,4,5,6 are expressed as percents)	Sex	Chemical	Dose (ug/L)	No. of Tanks	Mean	Median	Min	Max	Standard Deviation	Standard Error
			Ctrl	0.00	8	18.296	18.167	17.778	19.308	0.454	0.161
			ATZ	0.01	8	18.446	18.458	17.667	19.176	0.435	0.154
			ATZ	0.10	8	18.295	18.237	17.700	19.200	0.497	0.176
			ATZ	1.00	7	18.253	18.429	17.273	18.857	0.539	0.204
			ATZ	25.00	8	18.378	18.417	17.750	19.000	0.386	0.136
			ATZ	100.00	8	18.473	18.396	17.556	19.200	0.506	0.179
6	Gonadal image area	F	E2	0.20	8	1.358	1.356	1.263	1.525	0.078	0.027
			Ctrl	0.00	8	1.331	1.292	1.204	1.651	0.145	0.051
			ATZ	0.01	8	1.325	1.356	1.172	1.441	0.106	0.037
			ATZ	0.10	8	1.269	1.285	1.047	1.493	0.166	0.059
			ATZ	1.00	7	1.315	1.336	1.123	1.428	0.094	0.036
			ATZ	25.00	8	1.338	1.307	1.239	1.500	0.093	0.033
			ATZ	100.00	8	1.314	1.320	1.082	1.614	0.170	0.060
		M	E2	0.20	8	0.906	0.908	0.806	0.979	0.051	0.018
			Ctrl	0.00	8	0.839	0.820	0.783	0.917	0.047	0.017
			ATZ	0.01	8	0.876	0.876	0.805	0.926	0.038	0.014
			ATZ	0.10	8	0.831	0.831	0.784	0.905	0.040	0.014
			ATZ	1.00	7	0.836	0.838	0.789	0.898	0.039	0.015
			ATZ	25.00	8	0.861	0.848	0.801	0.932	0.052	0.019
			ATZ	100.00	8	0.897	0.890	0.833	0.988	0.054	0.019
7	Intersex (gross observation)	None	E2	0.20	8	0.000	0.000	0.000	0.000	0.000	0.000
			Ctrl	0.00	8	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	0.01	8	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	0.10	8	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	1.00	7	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	25.00	8	0.000	0.000	0.000	0.000	0.000	0.000

TABLE 2D.1. WILDLIFE GROSS - Basic Summary Statistics for all Response Variables

No.	Response Variable (all except #s 3,4,5,6 are expressed as percents)	Sex	Chemical	Dose (ug/L)	No. of Tanks	Mean	Median	Min	Max	Standard Deviation	Standard Error
			ATZ	100.00	8	0.000	0.000	0.000	0.000	0.000	0.000
8	Mixed tissue types (gross observation)	None	E2	0.20	8	2.063	2.000	0.000	4.167	2.206	0.780
			Ctrl	0.00	8	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	0.01	8	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	0.10	8	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	1.00	7	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	25.00	8	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	100.00	8	0.000	0.000	0.000	0.000	0.000	0.000
9	Gonadal adhesions	F	E2	0.20	8	0.000	0.000	0.000	0.000	0.000	0.000
			Ctrl	0.00	8	2.386	0.000	0.000	10.000	4.425	1.565
			ATZ	0.01	8	2.778	0.000	0.000	11.111	5.143	1.818
			ATZ	0.10	8	2.083	0.000	0.000	8.333	3.858	1.364
			ATZ	1.00	7	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	25.00	8	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	100.00	8	0.000	0.000	0.000	0.000	0.000	0.000
		M	E2	0.20	8	0.000	0.000	0.000	0.000	0.000	0.000
			Ctrl	0.00	8	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	0.01	8	0.781	0.000	0.000	6.250	2.210	0.781
			ATZ	0.10	8	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	1.00	7	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	25.00	8	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	100.00	8	0.000	0.000	0.000	0.000	0.000	0.000
10	Gonadal aplasia	F	E2	0.20	8	0.000	0.000	0.000	0.000	0.000	0.000
			Ctrl	0.00	8	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	0.01	8	0.893	0.000	0.000	7.143	2.525	0.893
			ATZ	0.10	8	0.000	0.000	0.000	0.000	0.000	0.000

TABLE 2D.1. WILDLIFE GROSS - Basic Summary Statistics for all Response Variables

No.	Response Variable (all except #s 3,4,5,6 are expressed as percents)	Sex	Chemical	Dose (ug/L)	No. of Tanks	Mean	Median	Min	Max	Standard Deviation	Standard Error
			ATZ	1.00	7	1.984	0.000	0.000	8.333	3.482	1.316
			ATZ	25.00	8	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	100.00	8	1.389	0.000	0.000	11.111	3.928	1.389
		M	E2	0.20	8	0.000	0.000	0.000	0.000	0.000	0.000
			Ctrl	0.00	8	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	0.01	8	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	0.10	8	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	1.00	7	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	25.00	8	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	100.00	8	0.000	0.000	0.000	0.000	0.000	0.000
11	Gonadal segmental aplasia	F	E2	0.20	8	2.064	0.000	0.000	6.250	2.870	1.015
			Ctrl	0.00	8	2.031	0.000	0.000	10.000	3.892	1.376
			ATZ	0.01	8	2.282	0.000	0.000	11.111	4.356	1.540
			ATZ	0.10	8	2.083	0.000	0.000	16.667	5.893	2.083
			ATZ	1.00	7	0.794	0.000	0.000	5.556	2.100	0.794
			ATZ	25.00	8	2.922	0.000	0.000	14.286	5.586	1.975
			ATZ	100.00	8	0.000	0.000	0.000	0.000	0.000	0.000
		M	E2	0.20	8	41.389	38.889	0.000	75.000	25.755	9.106
			Ctrl	0.00	8	25.736	27.885	11.111	42.857	10.726	3.792
			ATZ	0.01	8	24.495	21.591	7.692	58.333	15.485	5.475
			ATZ	0.10	8	29.979	32.500	0.000	50.000	16.785	5.935
			ATZ	1.00	7	14.933	13.333	9.091	33.333	8.415	3.180
			ATZ	25.00	8	23.881	22.500	9.091	42.857	11.677	4.129
			ATZ	100.00	8	19.724	18.015	9.091	35.714	10.278	3.634
12	Gonadal bifurcation	F	E2	0.20	8	0.000	0.000	0.000	0.000	0.000	0.000
			Ctrl	0.00	8	0.000	0.000	0.000	0.000	0.000	0.000

TABLE 2D.1. WILDLIFE GROSS - Basic Summary Statistics for all Response Variables

No.	Response Variable (all except #s 3,4,5,6 are expressed as percents)	Sex	Chemical	Dose (ug/L)	No. of Tanks	Mean	Median	Min	Max	Standard Deviation	Standard Error
			ATZ	0.01	8	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	0.10	8	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	1.00	7	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	25.00	8	0.962	0.000	0.000	7.692	2.720	0.962
			ATZ	100.00	8	0.000	0.000	0.000	0.000	0.000	0.000
		M	E2	0.20	8	6.250	0.000	0.000	25.000	11.573	4.092
			Ctrl	0.00	8	0.893	0.000	0.000	7.143	2.525	0.893
			ATZ	0.01	8	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	0.10	8	2.500	0.000	0.000	10.000	4.629	1.637
			ATZ	1.00	7	4.700	0.000	0.000	16.667	6.542	2.473
			ATZ	25.00	8	1.136	0.000	0.000	9.091	3.214	1.136
			ATZ	100.00	8	0.893	0.000	0.000	7.143	2.525	0.893
13	Gonadal deformity	F	E2	0.20	8	0.658	0.000	0.000	5.263	1.861	0.658
			Ctrl	0.00	8	2.386	0.000	0.000	10.000	4.425	1.565
			ATZ	0.01	8	1.136	0.000	0.000	9.091	3.214	1.136
			ATZ	0.10	8	6.951	6.971	0.000	16.667	6.935	2.452
			ATZ	1.00	7	0.952	0.000	0.000	6.667	2.520	0.952
			ATZ	25.00	8	1.726	0.000	0.000	7.143	3.199	1.131
			ATZ	100.00	8	2.976	0.000	0.000	11.111	4.383	1.550
		M	E2	0.20	8	0.000	0.000	0.000	0.000	0.000	0.000
			Ctrl	0.00	8	0.962	0.000	0.000	7.692	2.720	0.962
			ATZ	0.01	8	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	0.10	8	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	1.00	7	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	25.00	8	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	100.00	8	0.893	0.000	0.000	7.143	2.525	0.893

TABLE 2D.1. WILDLIFE GROSS - Basic Summary Statistics for all Response Variables

No.	Response Variable (all except #s 3,4,5,6 are expressed as percents)	Sex	Chemical	Dose (ug/L)	No. of Tanks	Mean	Median	Min	Max	Standard Deviation	Standard Error
14	Gonadal displacement	F	E2	0.20	8	0.000	0.000	0.000	0.000	0.000	0.000
			Ctrl	0.00	8	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	0.01	8	1.786	0.000	0.000	14.286	5.051	1.786
			ATZ	0.10	8	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	1.00	7	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	25.00	8	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	100.00	8	0.000	0.000	0.000	0.000	0.000	0.000
		M	E2	0.20	8	0.000	0.000	0.000	0.000	0.000	0.000
			Ctrl	0.00	8	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	0.01	8	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	0.10	8	2.639	0.000	0.000	11.111	4.895	1.731
			ATZ	1.00	7	5.374	0.000	0.000	16.667	7.350	2.778
			ATZ	25.00	8	1.250	0.000	0.000	10.000	3.536	1.250
			ATZ	100.00	8	0.000	0.000	0.000	0.000	0.000	0.000
15	Fused gonads	F	E2	0.20	8	0.893	0.000	0.000	7.143	2.525	0.893
			Ctrl	0.00	8	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	0.01	8	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	0.10	8	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	1.00	7	2.139	0.000	0.000	9.091	3.769	1.424
			ATZ	25.00	8	0.962	0.000	0.000	7.692	2.720	0.962
			ATZ	100.00	8	0.962	0.000	0.000	7.692	2.720	0.962
		M	E2	0.20	8	0.000	0.000	0.000	0.000	0.000	0.000
			Ctrl	0.00	8	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	0.01	8	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	0.10	8	1.042	0.000	0.000	8.333	2.946	1.042
			ATZ	1.00	7	0.000	0.000	0.000	0.000	0.000	0.000

TABLE 2D.1. WILDLIFE GROSS - Basic Summary Statistics for all Response Variables

No.	Response Variable (all except #s 3,4,5,6 are expressed as percents)	Sex	Chemical	Dose (ug/L)	No. of Tanks	Mean	Median	Min	Max	Standard Deviation	Standard Error
			ATZ	25.00	8	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	100.00	8	0.000	0.000	0.000	0.000	0.000	0.000
16	Gonadal hypertrophy	F	E2	0.20	8	1.316	0.000	0.000	5.263	2.436	0.861
			Ctrl	0.00	8	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	0.01	8	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	0.10	8	0.962	0.000	0.000	7.692	2.720	0.962
			ATZ	1.00	7	1.299	0.000	0.000	9.091	3.436	1.299
			ATZ	25.00	8	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	100.00	8	1.389	0.000	0.000	11.111	3.928	1.389
		M	E2	0.20	8	6.597	0.000	0.000	25.000	9.842	3.480
			Ctrl	0.00	8	6.368	7.418	0.000	16.667	6.053	2.140
			ATZ	0.01	8	3.079	0.000	0.000	12.500	4.691	1.659
			ATZ	0.10	8	0.962	0.000	0.000	7.692	2.720	0.962
			ATZ	1.00	7	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	25.00	8	1.786	0.000	0.000	7.143	3.307	1.169
			ATZ	100.00	8	2.273	0.000	0.000	9.091	4.208	1.488
17	Gonadal segmental hypertrophy	F	E2	0.20	8	0.000	0.000	0.000	0.000	0.000	0.000
			Ctrl	0.00	8	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	0.01	8	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	0.10	8	1.743	0.000	0.000	7.692	3.250	1.149
			ATZ	1.00	7	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	25.00	8	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	100.00	8	0.000	0.000	0.000	0.000	0.000	0.000
		M	E2	0.20	8	3.889	0.000	0.000	20.000	7.583	2.681
			Ctrl	0.00	8	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	0.01	8	0.962	0.000	0.000	7.692	2.720	0.962

TABLE 2D.1. WILDLIFE GROSS - Basic Summary Statistics for all Response Variables

No.	Response Variable (all except #s 3,4,5,6 are expressed as percents)	Sex	Chemical	Dose (ug/L)	No. of Tanks	Mean	Median	Min	Max	Standard Deviation	Standard Error
			ATZ	0.10	8	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	1.00	7	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	25.00	8	2.386	0.000	0.000	10.000	4.425	1.565
			ATZ	100.00	8	0.893	0.000	0.000	7.143	2.525	0.893
18	Gonadal hypoplasia	F	E2	0.20	8	36.961	33.289	21.053	55.000	10.797	3.817
			Ctrl	0.00	8	32.263	30.303	10.000	54.545	16.691	5.901
			ATZ	0.01	8	27.022	28.571	8.333	41.667	10.542	3.727
			ATZ	0.10	8	33.783	35.897	18.182	50.000	12.840	4.539
			ATZ	1.00	7	39.367	40.000	30.000	50.000	6.162	2.329
			ATZ	25.00	8	29.612	30.385	0.000	50.000	14.931	5.279
			ATZ	100.00	8	33.086	30.000	21.429	62.500	13.540	4.787
		M	E2	0.20	8	12.014	0.000	0.000	60.000	21.360	7.552
			Ctrl	0.00	8	1.923	0.000	0.000	7.692	3.561	1.259
			ATZ	0.01	8	1.970	0.000	0.000	9.091	3.704	1.310
			ATZ	0.10	8	11.626	13.248	0.000	20.000	8.270	2.924
			ATZ	1.00	7	4.444	0.000	0.000	20.000	8.012	3.028
			ATZ	25.00	8	5.214	7.143	0.000	10.000	4.419	1.562
			ATZ	100.00	8	3.926	0.000	0.000	12.500	5.636	1.993
19	Gonadal segmental hypoplasia	F	E2	0.20	8	14.692	13.750	7.143	25.000	5.910	2.090
			Ctrl	0.00	8	11.638	10.795	0.000	20.000	7.030	2.485
			ATZ	0.01	8	18.141	15.476	0.000	44.444	14.696	5.196
			ATZ	0.10	8	9.930	8.333	6.250	16.667	3.864	1.366
			ATZ	1.00	7	9.642	9.091	0.000	17.647	5.372	2.030
			ATZ	25.00	8	16.411	20.714	0.000	25.000	9.479	3.351
			ATZ	100.00	8	16.481	11.905	0.000	46.154	14.827	5.242
		M	E2	0.20	8	9.028	0.000	0.000	33.333	13.260	4.688

TABLE 2D.1. WILDLIFE GROSS - Basic Summary Statistics for all Response Variables

No.	Response Variable (all except #s 3,4,5,6 are expressed as percents)	Sex	Chemical	Dose (ug/L)	No. of Tanks	Mean	Median	Min	Max	Standard Deviation	Standard Error
			Ctrl	0.00	8	4.274	0.000	0.000	15.385	6.247	2.209
			ATZ	0.01	8	2.965	0.000	0.000	8.333	4.097	1.448
			ATZ	0.10	8	5.684	8.013	0.000	11.111	4.827	1.706
			ATZ	1.00	7	4.185	0.000	0.000	11.111	5.263	1.989
			ATZ	25.00	8	4.951	3.571	0.000	14.286	5.660	2.001
			ATZ	100.00	8	1.786	0.000	0.000	14.286	5.051	1.786
20	Gonadal translucence	F	E2	0.20	8	20.204	15.395	0.000	42.105	14.263	5.043
			Ctrl	0.00	8	11.671	12.500	0.000	18.182	6.362	2.249
			ATZ	0.01	8	10.539	11.111	0.000	25.000	8.480	2.998
			ATZ	0.10	8	13.851	15.385	0.000	25.000	7.438	2.630
			ATZ	1.00	7	21.792	25.000	9.091	36.364	9.998	3.779
			ATZ	25.00	8	14.755	15.000	0.000	30.769	13.651	4.826
			ATZ	100.00	8	15.012	12.917	7.143	28.571	7.478	2.644
		M	E2	0.20	8	0.000	0.000	0.000	0.000	0.000	0.000
			Ctrl	0.00	8	2.976	0.000	0.000	16.667	6.070	2.146
			ATZ	0.01	8	1.517	0.000	0.000	6.250	2.810	0.993
			ATZ	0.10	8	3.045	0.000	0.000	8.333	4.207	1.487
			ATZ	1.00	7	2.251	0.000	0.000	9.091	3.908	1.477
			ATZ	25.00	8	1.786	0.000	0.000	14.286	5.051	1.786
			ATZ	100.00	8	2.699	0.000	0.000	12.500	5.080	1.796
21	Gonadal segmental translucence	F	E2	0.20	8	4.663	5.263	0.000	10.526	4.331	1.531
			Ctrl	0.00	8	0.781	0.000	0.000	6.250	2.210	0.781
			ATZ	0.01	8	4.059	2.941	0.000	11.111	4.579	1.619
			ATZ	0.10	8	6.791	7.692	0.000	8.333	2.828	1.000
			ATZ	1.00	7	3.997	0.000	0.000	13.333	5.469	2.067
			ATZ	25.00	8	7.598	7.692	0.000	12.500	3.603	1.274

TABLE 2D.1. WILDLIFE GROSS - Basic Summary Statistics for all Response Variables

No.	Response Variable (all except #s 3,4,5,6 are expressed as percents)	Sex	Chemical	Dose (ug/L)	No. of Tanks	Mean	Median	Min	Max	Standard Deviation	Standard Error
			ATZ	100.00	8	5.305	6.111	0.000	15.385	5.305	1.876
		M	E2	0.20	8	8.750	0.000	0.000	33.333	12.964	4.583
			Ctrl	0.00	8	0.893	0.000	0.000	7.143	2.525	0.893
			ATZ	0.01	8	2.576	0.000	0.000	7.692	3.577	1.265
			ATZ	0.10	8	1.250	0.000	0.000	10.000	3.536	1.250
			ATZ	1.00	7	2.886	0.000	0.000	11.111	4.963	1.876
			ATZ	25.00	8	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	100.00	8	9.373	10.101	0.000	14.286	4.402	1.556
22	Gonadal pearling	F	E2	0.20	8	0.000	0.000	0.000	0.000	0.000	0.000
			Ctrl	0.00	8	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	0.01	8	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	0.10	8	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	1.00	7	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	25.00	8	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	100.00	8	0.000	0.000	0.000	0.000	0.000	0.000
		M	E2	0.20	8	2.778	0.000	0.000	22.222	7.857	2.778
			Ctrl	0.00	8	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	0.01	8	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	0.10	8	1.042	0.000	0.000	8.333	2.946	1.042
			ATZ	1.00	7	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	25.00	8	1.136	0.000	0.000	9.091	3.214	1.136
			ATZ	100.00	8	0.000	0.000	0.000	0.000	0.000	0.000
23	Melanophores decreased in ovaries	F	E2	0.20	8	0.000	0.000	0.000	0.000	0.000	0.000
			Ctrl	0.00	8	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	0.01	8	1.786	0.000	0.000	14.286	5.051	1.786
			ATZ	0.10	8	0.000	0.000	0.000	0.000	0.000	0.000

TABLE 2D.1. WILDLIFE GROSS - Basic Summary Statistics for all Response Variables

No.	Response Variable (all except #s 3,4,5,6 are expressed as percents)	Sex	Chemical	Dose (ug/L)	No. of Tanks	Mean	Median	Min	Max	Standard Deviation	Standard Error
			ATZ	1.00	7	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	25.00	8	1.042	0.000	0.000	8.333	2.946	1.042
			ATZ	100.00	8	0.000	0.000	0.000	0.000	0.000	0.000
24	Internal melanophores in testes	M	E2	0.20	8	6.250	0.000	0.000	25.000	11.573	4.092
			Ctrl	0.00	8	2.965	0.000	0.000	8.333	4.097	1.448
			ATZ	0.01	8	0.962	0.000	0.000	7.692	2.720	0.962
			ATZ	0.10	8	1.250	0.000	0.000	10.000	3.536	1.250
			ATZ	1.00	7	1.299	0.000	0.000	9.091	3.436	1.299
			ATZ	25.00	8	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	100.00	8	1.389	0.000	0.000	11.111	3.928	1.389
25	Frequency of males among just males and females	None	E2	0.20	8	19.667	17.391	9.524	39.130	9.379	3.316
			Ctrl	0.00	8	49.264	53.170	27.273	60.000	11.471	4.056
			ATZ	0.01	8	53.688	53.083	32.000	70.833	12.299	4.348
			ATZ	0.10	8	46.050	46.640	36.000	54.167	5.385	1.904
			ATZ	1.00	7	43.630	47.826	26.087	60.000	13.347	5.045
			ATZ	25.00	8	49.032	47.917	34.783	63.636	9.168	3.241
			ATZ	100.00	8	45.506	43.739	28.000	68.000	13.107	4.634
26	Frequency of males	None	E2	0.20	8	18.526	16.000	8.333	37.500	9.089	3.214
			Ctrl	0.00	8	49.264	53.170	27.273	60.000	11.471	4.056
			ATZ	0.01	8	53.688	53.083	32.000	70.833	12.299	4.348
			ATZ	0.10	8	46.050	46.640	36.000	54.167	5.385	1.904
			ATZ	1.00	7	43.630	47.826	26.087	60.000	13.347	5.045
			ATZ	25.00	8	49.032	47.917	34.783	63.636	9.168	3.241
			ATZ	100.00	8	45.506	43.739	28.000	68.000	13.107	4.634
27	Frequency of females	None	E2	0.20	8	75.307	76.000	58.333	86.957	9.097	3.216

TABLE 2D.1. WILDLIFE GROSS - Basic Summary Statistics for all Response Variables

No.	Response Variable (all except #s 3,4,5,6 are expressed as percents)	Sex	Chemical	Dose (ug/L)	No. of Tanks	Mean	Median	Min	Max	Standard Deviation	Standard Error
			Ctrl	0.00	8	50.736	46.830	40.000	72.727	11.471	4.056
			ATZ	0.01	8	46.313	46.917	29.167	68.000	12.299	4.348
			ATZ	0.10	8	53.950	53.360	45.833	64.000	5.385	1.904
			ATZ	1.00	7	56.370	52.174	40.000	73.913	13.347	5.045
			ATZ	25.00	8	50.968	52.083	36.364	65.217	9.168	3.241
			ATZ	100.00	8	54.494	56.261	32.000	72.000	13.107	4.634
28	Frequency of intersex	None	E2	0.20	8	0.000	0.000	0.000	0.000	0.000	0.000
			Ctrl	0.00	8	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	0.01	8	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	0.10	8	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	1.00	7	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	25.00	8	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	100.00	8	0.000	0.000	0.000	0.000	0.000	0.000
29	Frequency of mixed sex	None	E2	0.20	8	6.167	6.083	0.000	12.500	4.943	1.747
			Ctrl	0.00	8	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	0.01	8	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	0.10	8	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	1.00	7	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	25.00	8	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	100.00	8	0.000	0.000	0.000	0.000	0.000	0.000

TABLE 2D.2. WILDLIFE EPL- Basic Summary Statistics for all Response Variables

No.	Response Variable (Grade>0 represents % in tank having any size effect; Grade>1 represents % in tank having effect with a severity level grade >1)	Sex	Chemical	Dose (ug/L)	No. of Tanks	Mean (%)	Median (%)	Min (%)	Max (%)	Standard Deviation	Standard Error
1	Segmental hypoplasia (Grade>0)	F	E2	0.20	8	0.000	0.000	0.000	0.000	0.000	0.000
			Ctrl	0.00	8	2.813	0.000	0.000	12.500	5.250	1.856
			ATZ	0.01	8	1.935	0.000	0.000	8.333	3.596	1.271
			ATZ	0.10	8	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	1.00	7	0.840	0.000	0.000	5.882	2.223	0.840
			ATZ	25.00	8	2.029	0.000	0.000	9.091	3.793	1.341
			ATZ	100.00	8	0.962	0.000	0.000	7.692	2.720	0.962
		M	E2	0.20	8	33.819	36.667	0.000	55.556	19.087	6.748
			Ctrl	0.00	8	26.393	23.077	15.385	50.000	11.347	4.012
			ATZ	0.01	8	9.588	10.096	0.000	25.000	9.360	3.309
			ATZ	0.10	8	16.181	18.333	0.000	44.444	14.283	5.050
			ATZ	1.00	7	8.930	9.091	0.000	18.182	7.160	2.706
			ATZ	25.00	8	15.137	9.545	0.000	45.455	14.682	5.191
			ATZ	100.00	8	14.403	13.025	0.000	28.571	8.698	3.075
	Segmental hypoplasia (Grade>1)	F	E2	0.20	8	0.000	0.000	0.000	0.000	0.000	0.000
			Ctrl	0.00	8	1.563	0.000	0.000	12.500	4.419	1.563
			ATZ	0.01	8	1.042	0.000	0.000	8.333	2.946	1.042
			ATZ	0.10	8	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	1.00	7	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	25.00	8	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	100.00	8	0.000	0.000	0.000	0.000	0.000	0.000
		M	E2	0.20	8	8.750	0.000	0.000	25.000	12.174	4.304

TABLE 2D.2. WILDLIFE EPL- Basic Summary Statistics for all Response Variables

No.	Response Variable (Grade>0 represents % in tank having any size effect; Grade>1 represents % in tank having effect with a severity level grade >1)	Sex	Chemical	Dose (ug/L)	No. of Tanks	Mean (%)	Median (%)	Min (%)	Max (%)	Standard Deviation	Standard Error
			Ctrl	0.00	8	1.854	0.000	0.000	7.692	3.437	1.215
			ATZ	0.01	8	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	0.10	8	3.889	0.000	0.000	11.111	5.378	1.901
			ATZ	1.00	7	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	25.00	8	1.136	0.000	0.000	9.091	3.214	1.136
			ATZ	100.00	8	1.628	0.000	0.000	7.143	3.034	1.073
2	Segmental aplasia (Grade>0)	F	E2	0.20	8	0.000	0.000	0.000	0.000	0.000	0.000
			Ctrl	0.00	8	2.031	0.000	0.000	10.000	3.892	1.376
			ATZ	0.01	8	1.389	0.000	0.000	11.111	3.928	1.389
			ATZ	0.10	8	2.083	0.000	0.000	16.667	5.893	2.083
			ATZ	1.00	7	0.794	0.000	0.000	5.556	2.100	0.794
			ATZ	25.00	8	0.893	0.000	0.000	7.143	2.525	0.893
			ATZ	100.00	8	0.000	0.000	0.000	0.000	0.000	0.000
		M	E2	0.20	8	9.514	0.000	0.000	40.000	15.233	5.386
			Ctrl	0.00	8	4.602	0.000	0.000	21.429	8.672	3.066
			ATZ	0.01	8	4.607	0.000	0.000	16.667	6.797	2.403
			ATZ	0.10	8	10.972	9.167	0.000	30.000	9.994	3.534
			ATZ	1.00	7	2.540	0.000	0.000	11.111	4.523	1.710
			ATZ	25.00	8	11.112	12.143	0.000	21.429	8.047	2.845
			ATZ	100.00	8	1.872	0.000	0.000	9.091	3.570	1.262
	Segmental aplasia (Grade>1)	F	E2	0.20	8	0.000	0.000	0.000	0.000	0.000	0.000
			Ctrl	0.00	8	1.250	0.000	0.000	10.000	3.536	1.250

TABLE 2D.2. WILDLIFE EPL- Basic Summary Statistics for all Response Variables

No.	Response Variable (Grade>0 represents % in tank having any size effect; Grade>1 represents % in tank having effect with a severity level grade >1)	Sex	Chemical	Dose (ug/L)	No. of Tanks	Mean (%)	Median (%)	Min (%)	Max (%)	Standard Deviation	Standard Error
			ATZ	0.01	8	1.389	0.000	0.000	11.111	3.928	1.389
			ATZ	0.10	8	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	1.00	7	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	25.00	8	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	100.00	8	0.000	0.000	0.000	0.000	0.000	0.000
		M	E2	0.20	8	6.389	0.000	0.000	40.000	14.127	4.994
			Ctrl	0.00	8	0.893	0.000	0.000	7.143	2.525	0.893
			ATZ	0.01	8	2.083	0.000	0.000	16.667	5.893	2.083
			ATZ	0.10	8	4.931	0.000	0.000	20.000	7.544	2.667
			ATZ	1.00	7	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	25.00	8	2.976	0.000	0.000	8.333	4.124	1.458
			ATZ	100.00	8	0.000	0.000	0.000	0.000	0.000	0.000
3	Decreased germ cells in gonad (Grade>0)	F	E2	0.20	8	57.185	57.895	36.842	71.429	11.733	4.148
			Ctrl	0.00	8	50.402	52.273	31.250	58.333	8.295	2.933
			ATZ	0.01	8	53.520	51.471	41.667	71.429	10.679	3.776
			ATZ	0.10	8	61.640	56.090	38.462	91.667	17.653	6.241
			ATZ	1.00	7	54.017	54.545	40.000	66.667	9.833	3.717
			ATZ	25.00	8	54.420	57.273	28.571	69.231	13.847	4.896
			ATZ	100.00	8	57.772	58.547	35.714	77.778	14.712	5.201
		M	E2	0.20	8	68.681	70.833	33.333	100.000	20.136	7.119
			Ctrl	0.00	8	80.838	83.333	66.667	92.857	10.140	3.585
			ATZ	0.01	8	81.927	83.974	68.750	93.333	8.396	2.968
			ATZ	0.10	8	84.889	86.667	61.538	100.000	11.281	3.988

TABLE 2D.2. WILDLIFE EPL- Basic Summary Statistics for all Response Variables

No.	Response Variable (Grade>0 represents % in tank having any size effect; Grade>1 represents % in tank having effect with a severity level grade >1)	Sex	Chemical	Dose (ug/L)	No. of Tanks	Mean (%)	Median (%)	Min (%)	Max (%)	Standard Deviation	Standard Error
			ATZ	1.00	7	83.999	85.714	63.636	100.000	11.598	4.384
			ATZ	25.00	8	78.162	82.576	54.545	87.500	11.555	4.085
			ATZ	100.00	8	82.895	80.195	70.588	100.000	9.169	3.242
	Decreased germ cells in gonad (Grade>1)	F	E2	0.20	8	43.475	38.750	26.316	63.158	12.230	4.324
			Ctrl	0.00	8	33.291	33.636	18.182	50.000	12.461	4.406
			ATZ	0.01	8	36.334	34.874	16.667	66.667	16.153	5.711
			ATZ	0.10	8	44.759	51.923	12.500	75.000	20.740	7.333
			ATZ	1.00	7	37.203	36.364	20.000	54.545	10.512	3.973
			ATZ	25.00	8	31.523	34.848	0.000	61.538	18.654	6.595
			ATZ	100.00	8	44.650	47.222	21.429	61.111	13.683	4.838
		M	E2	0.20	8	63.125	66.667	33.333	100.000	23.170	8.192
			Ctrl	0.00	8	53.507	56.090	33.333	71.429	13.616	4.814
			ATZ	0.01	8	54.173	54.412	25.000	73.333	15.267	5.398
			ATZ	0.10	8	64.955	66.667	46.154	81.818	12.890	4.557
			ATZ	1.00	7	57.937	55.556	42.857	83.333	13.676	5.169
			ATZ	25.00	8	55.214	55.000	41.667	66.667	9.565	3.382
			ATZ	100.00	8	66.809	64.171	50.000	100.000	15.869	5.611
4	Dilated testis tubules (Grade>0)	M	E2	0.20	8	73.889	78.889	0.000	100.000	31.549	11.154
			Ctrl	0.00	8	11.405	9.722	0.000	23.077	7.159	2.531
			ATZ	0.01	8	7.747	4.545	0.000	25.000	9.415	3.329
			ATZ	0.10	8	6.681	8.392	0.000	16.667	6.124	2.165
			ATZ	1.00	7	4.966	0.000	0.000	14.286	6.586	2.489

TABLE 2D.2. WILDLIFE EPL- Basic Summary Statistics for all Response Variables

No.	Response Variable (Grade>0 represents % in tank having any size effect; Grade>1 represents % in tank having effect with a severity level grade >1)	Sex	Chemical	Dose (ug/L)	No. of Tanks	Mean (%)	Median (%)	Min (%)	Max (%)	Standard Deviation	Standard Error
			ATZ	25.00	8	5.993	3.571	0.000	18.182	7.249	2.563
			ATZ	100.00	8	16.226	14.286	9.091	27.273	6.674	2.360
	Dilated testis tubules (Grade>1)	M	E2	0.20	8	46.389	50.000	0.000	66.667	21.908	7.746
			Ctrl	0.00	8	0.962	0.000	0.000	7.692	2.720	0.962
			ATZ	0.01	8	0.962	0.000	0.000	7.692	2.720	0.962
			ATZ	0.10	8	2.212	0.000	0.000	10.000	4.141	1.464
			ATZ	1.00	7	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	25.00	8	2.273	0.000	0.000	18.182	6.428	2.273
			ATZ	100.00	8	6.133	0.000	0.000	25.000	9.923	3.508
5	Dividing gonocytes in testes (Grade>0)	M	E2	0.20	8	40.764	42.222	0.000	66.667	22.061	7.800
			Ctrl	0.00	8	9.066	7.738	0.000	25.000	9.277	3.280
			ATZ	0.01	8	17.198	16.516	0.000	37.500	11.468	4.055
			ATZ	0.10	8	22.077	19.872	9.091	40.000	10.658	3.768
			ATZ	1.00	7	14.129	14.286	7.143	18.182	4.039	1.526
			ATZ	25.00	8	17.909	18.333	0.000	28.571	9.692	3.427
			ATZ	100.00	8	16.140	13.025	0.000	28.571	9.933	3.512
	Dividing gonocytes in testes (Grade>1)	M	E2	0.20	8	13.889	0.000	0.000	66.667	24.307	8.594
			Ctrl	0.00	8	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	0.01	8	0.962	0.000	0.000	7.692	2.720	0.962
			ATZ	0.10	8	5.139	0.000	0.000	20.000	7.674	2.713
			ATZ	1.00	7	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	25.00	8	0.893	0.000	0.000	7.143	2.525	0.893

TABLE 2D.2. WILDLIFE EPL- Basic Summary Statistics for all Response Variables

No.	Response Variable (Grade>0 represents % in tank having any size effect; Grade>1 represents % in tank having effect with a severity level grade >1)	Sex	Chemical	Dose (ug/L)	No. of Tanks	Mean (%)	Median (%)	Min (%)	Max (%)	Standard Deviation	Standard Error
			ATZ	100.00	8	0.000	0.000	0.000	0.000	0.000	0.000
6	Internal melanophores in testis (Grade>0)	M	E2	0.20	8	100.000	100.000	100.000	100.000	0.000	0.000
			Ctrl	0.00	8	80.750	77.350	61.538	100.000	13.418	4.744
			ATZ	0.01	8	73.643	73.756	54.545	100.000	14.840	5.247
			ATZ	0.10	8	77.076	80.909	55.556	90.000	12.173	4.304
			ATZ	1.00	7	64.651	66.667	42.857	83.333	14.050	5.310
			ATZ	25.00	8	66.465	64.583	40.000	92.857	18.307	6.473
			ATZ	100.00	8	76.935	72.078	62.500	100.000	13.231	4.678
	Internal melanophores in testis (Grade>1)	M	E2	0.20	8	58.472	50.000	33.333	100.000	26.280	9.291
			Ctrl	0.00	8	15.995	12.698	0.000	30.769	11.513	4.071
			ATZ	0.01	8	14.452	13.369	0.000	30.769	10.352	3.660
			ATZ	0.10	8	18.676	16.667	7.692	30.000	9.193	3.250
			ATZ	1.00	7	13.041	16.667	0.000	20.000	7.337	2.773
			ATZ	25.00	8	10.983	9.545	0.000	21.429	6.573	2.324
			ATZ	100.00	8	13.622	15.341	0.000	33.333	12.685	4.485
7	Decreased ovarian pigmentation (Grade>0)	F	E2	0.20	8	0.000	0.000	0.000	0.000	0.000	0.000
			Ctrl	0.00	8	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	0.01	8	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	0.10	8	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	1.00	7	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	25.00	8	1.042	0.000	0.000	8.333	2.946	1.042
			ATZ	100.00	8	0.000	0.000	0.000	0.000	0.000	0.000

TABLE 2D.2. WILDLIFE EPL- Basic Summary Statistics for all Response Variables

No.	Response Variable (Grade>0 represents % in tank having any size effect; Grade>1 represents % in tank having effect with a severity level grade >1)	Sex	Chemical	Dose (ug/L)	No. of Tanks	Mean (%)	Median (%)	Min (%)	Max (%)	Standard Deviation	Standard Error
	Decreased ovarian pigmentation (Grade>1)	F	E2	0.20	8	0.000	0.000	0.000	0.000	0.000	0.000
			Ctrl	0.00	8	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	0.01	8	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	0.10	8	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	1.00	7	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	25.00	8	1.042	0.000	0.000	8.333	2.946	1.042
			ATZ	100.00	8	0.000	0.000	0.000	0.000	0.000	0.000
8	Decreased ovarian cavity size (Grade>0)	F	E2	0.20	8	26.262	23.026	12.500	42.857	12.190	4.310
			Ctrl	0.00	8	14.432	18.466	0.000	27.273	10.848	3.835
			ATZ	0.01	8	6.791	8.333	0.000	14.286	6.103	2.158
			ATZ	0.10	8	13.931	15.341	0.000	25.000	10.528	3.722
			ATZ	1.00	7	15.244	13.333	9.091	25.000	6.742	2.548
			ATZ	25.00	8	18.163	19.048	7.692	27.273	7.566	2.675
			ATZ	100.00	8	12.359	11.111	0.000	25.000	8.072	2.854
	Decreased ovarian cavity size (Grade>1)	F	E2	0.20	8	10.922	12.406	0.000	21.053	6.767	2.392
			Ctrl	0.00	8	4.616	0.000	0.000	18.750	7.034	2.487
			ATZ	0.01	8	3.869	0.000	0.000	14.286	5.647	1.996
			ATZ	0.10	8	3.921	3.125	0.000	9.091	4.265	1.508
			ATZ	1.00	7	6.536	0.000	0.000	23.529	9.689	3.662
			ATZ	25.00	8	6.741	7.418	0.000	20.000	6.859	2.425
			ATZ	100.00	8	1.587	0.000	0.000	7.143	2.970	1.050

TABLE 2D.2. WILDLIFE EPL- Basic Summary Statistics for all Response Variables

No.	Response Variable (Grade>0 represents % in tank having any size effect; Grade>1 represents % in tank having effect with a severity level grade >1)	Sex	Chemical	Dose (ug/L)	No. of Tanks	Mean (%)	Median (%)	Min (%)	Max (%)	Standard Deviation	Standard Error
9	Increased ovarian cavity size (Grade>0)	F	E2	0.20	8	11.488	10.263	0.000	31.579	10.561	3.734
			Ctrl	0.00	8	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	0.01	8	0.735	0.000	0.000	5.882	2.080	0.735
			ATZ	0.10	8	1.743	0.000	0.000	7.692	3.250	1.149
			ATZ	1.00	7	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	25.00	8	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	100.00	8	0.893	0.000	0.000	7.143	2.525	0.893
	Increased ovarian cavity size (Grade>1)	F	E2	0.20	8	2.632	0.000	0.000	10.526	3.979	1.407
			Ctrl	0.00	8	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	0.01	8	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	0.10	8	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	1.00	7	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	25.00	8	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	100.00	8	0.893	0.000	0.000	7.143	2.525	0.893
10	Ovarian interstitial fibrosis (Grade>0)	F	E2	0.20	8	10.452	12.763	0.000	15.789	6.002	2.122
			Ctrl	0.00	8	8.262	3.125	0.000	25.000	10.186	3.601
			ATZ	0.01	8	15.166	15.278	0.000	36.364	14.811	5.236
			ATZ	0.10	8	8.523	4.167	0.000	33.333	11.949	4.225
			ATZ	1.00	7	15.368	11.765	8.333	27.273	7.125	2.693
			ATZ	25.00	8	9.878	11.250	0.000	16.667	6.648	2.350
			ATZ	100.00	8	19.088	18.803	7.143	37.500	10.721	3.790

TABLE 2D.2. WILDLIFE EPL- Basic Summary Statistics for all Response Variables

No.	Response Variable (Grade>0 represents % in tank having any size effect; Grade>1 represents % in tank having effect with a severity level grade >1)	Sex	Chemical	Dose (ug/L)	No. of Tanks	Mean (%)	Median (%)	Min (%)	Max (%)	Standard Deviation	Standard Error
	Ovarian interstitial fibrosis (Grade>1)	F	E2	0.20	8	1.908	0.000	0.000	10.000	3.753	1.327
			Ctrl	0.00	8	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	0.01	8	5.691	2.941	0.000	22.222	7.759	2.743
			ATZ	0.10	8	2.083	0.000	0.000	8.333	3.858	1.364
			ATZ	1.00	7	3.918	0.000	0.000	10.000	4.910	1.856
			ATZ	25.00	8	1.042	0.000	0.000	8.333	2.946	1.042
			ATZ	100.00	8	3.671	0.000	0.000	11.111	5.212	1.843
11	Mononuclear cell infiltrates in ovary (Grade>0)	F	E2	0.20	8	20.092	21.053	10.526	30.000	7.027	2.485
			Ctrl	0.00	8	20.705	18.333	6.250	37.500	11.942	4.222
			ATZ	0.01	8	23.897	26.786	0.000	35.294	11.151	3.942
			ATZ	0.10	8	24.548	21.591	8.333	41.667	12.515	4.425
			ATZ	1.00	7	18.837	13.333	9.091	36.364	11.873	4.488
			ATZ	25.00	8	21.239	21.538	0.000	46.154	16.106	5.694
			ATZ	100.00	8	17.445	17.857	0.000	38.462	12.488	4.415
	Mononuclear cell infiltrates in ovary (Grade>1)	F	E2	0.20	8	0.658	0.000	0.000	5.263	1.861	0.658
			Ctrl	0.00	8	1.136	0.000	0.000	9.091	3.214	1.136
			ATZ	0.01	8	1.786	0.000	0.000	14.286	5.051	1.786
			ATZ	0.10	8	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	1.00	7	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	25.00	8	3.357	0.000	0.000	12.500	4.924	1.741
			ATZ	100.00	8	0.000	0.000	0.000	0.000	0.000	0.000

TABLE 2D.2. WILDLIFE EPL- Basic Summary Statistics for all Response Variables

No.	Response Variable (Grade>0 represents % in tank having any size effect; Grade>1 represents % in tank having effect with a severity level grade >1)	Sex	Chemical	Dose (ug/L)	No. of Tanks	Mean (%)	Median (%)	Min (%)	Max (%)	Standard Deviation	Standard Error
12	One or more immature ovaries	F	E2	0.20	8	4.149	5.132	0.000	10.526	3.863	1.366
			Ctrl	0.00	8	4.209	3.125	0.000	10.000	4.620	1.633
			ATZ	0.01	8	1.042	0.000	0.000	8.333	2.946	1.042
			ATZ	0.10	8	3.045	0.000	0.000	8.333	4.207	1.487
			ATZ	1.00	7	0.794	0.000	0.000	5.556	2.100	0.794
			ATZ	25.00	8	6.229	6.905	0.000	20.000	6.694	2.367
			ATZ	100.00	8	0.000	0.000	0.000	0.000	0.000	0.000
	One or more unable to stage ovaries	F	E2	0.20	8	21.187	18.750	10.526	36.842	11.473	4.056
			Ctrl	0.00	8	14.910	14.583	0.000	37.500	11.097	3.923
			ATZ	0.01	8	15.346	13.369	0.000	33.333	11.087	3.920
			ATZ	0.10	8	18.468	16.026	6.250	36.364	9.952	3.518
			ATZ	1.00	7	11.803	10.000	0.000	23.529	7.758	2.932
			ATZ	25.00	8	13.202	17.424	0.000	30.769	11.715	4.142
			ATZ	100.00	8	18.947	18.803	7.143	33.333	10.053	3.554
13	Fused kidneys (Grade>0)	F	E2	0.20	8	17.091	18.026	5.000	28.571	9.477	3.351
			Ctrl	0.00	8	15.900	16.250	0.000	33.333	12.515	4.425
			ATZ	0.01	8	22.115	24.747	0.000	41.667	12.782	4.519
			ATZ	0.10	8	21.687	25.000	0.000	45.455	15.144	5.354
			ATZ	1.00	7	18.563	13.333	8.333	36.364	10.632	4.019
			ATZ	25.00	8	20.274	21.329	0.000	38.462	13.626	4.818
			ATZ	100.00	8	26.889	24.038	5.556	44.444	13.933	4.926
		M	E2	0.20	8	3.472	0.000	0.000	16.667	6.599	2.333

TABLE 2D.2. WILDLIFE EPL- Basic Summary Statistics for all Response Variables

No.	Response Variable (Grade>0 represents % in tank having any size effect; Grade>1 represents % in tank having effect with a severity level grade >1)	Sex	Chemical	Dose (ug/L)	No. of Tanks	Mean (%)	Median (%)	Min (%)	Max (%)	Standard Deviation	Standard Error
			Ctrl	0.00	8	10.600	8.333	0.000	30.769	9.905	3.502
			ATZ	0.01	8	7.012	8.013	0.000	17.647	6.624	2.342
			ATZ	0.10	8	6.279	4.167	0.000	18.182	7.471	2.641
			ATZ	1.00	7	20.705	14.286	9.091	33.333	10.277	3.884
			ATZ	25.00	8	12.120	13.393	0.000	18.182	6.270	2.217
			ATZ	100.00	8	16.323	13.393	9.091	33.333	8.024	2.837
	Fused kidneys (Grade>1)	F	E2	0.20	8	0.000	0.000	0.000	0.000	0.000	0.000
			Ctrl	0.00	8	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	0.01	8	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	0.10	8	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	1.00	7	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	25.00	8	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	100.00	8	0.000	0.000	0.000	0.000	0.000	0.000
		M	E2	0.20	8	0.000	0.000	0.000	0.000	0.000	0.000
			Ctrl	0.00	8	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	0.01	8	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	0.10	8	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	1.00	7	0.952	0.000	0.000	6.667	2.520	0.952
			ATZ	25.00	8	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	100.00	8	0.000	0.000	0.000	0.000	0.000	0.000
14	Mineralization (Grade>0)	F	E2	0.20	8	12.695	13.750	0.000	26.316	9.287	3.284
			Ctrl	0.00	8	13.556	10.795	6.250	25.000	6.714	2.374
			ATZ	0.01	8	10.535	10.101	0.000	17.647	5.757	2.035
			ATZ	0.10	8	9.475	8.013	0.000	18.750	7.336	2.594

TABLE 2D.2. WILDLIFE EPL- Basic Summary Statistics for all Response Variables

No.	Response Variable (Grade>0 represents % in tank having any size effect; Grade>1 represents % in tank having effect with a severity level grade >1)	Sex	Chemical	Dose (ug/L)	No. of Tanks	Mean (%)	Median (%)	Min (%)	Max (%)	Standard Deviation	Standard Error
			ATZ	1.00	7	16.984	16.667	6.667	30.000	8.994	3.399
			ATZ	25.00	8	13.415	10.476	0.000	36.364	14.047	4.966
			ATZ	100.00	8	8.980	6.250	0.000	30.769	11.123	3.933
		M	E2	0.20	8	6.250	0.000	0.000	50.000	17.678	6.250
			Ctrl	0.00	8	9.730	11.538	0.000	21.429	8.890	3.143
			ATZ	0.01	8	13.297	16.250	0.000	27.273	11.782	4.165
			ATZ	0.10	8	11.995	13.889	0.000	30.000	11.217	3.966
			ATZ	1.00	7	18.256	16.667	7.143	33.333	9.464	3.577
			ATZ	25.00	8	10.666	9.091	0.000	25.000	7.353	2.600
			ATZ	100.00	8	17.690	14.545	0.000	37.500	14.134	4.997
	Mineralization (Grade>1)	F	E2	0.20	8	0.000	0.000	0.000	0.000	0.000	0.000
			Ctrl	0.00	8	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	0.01	8	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	0.10	8	1.042	0.000	0.000	8.333	2.946	1.042
			ATZ	1.00	7	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	25.00	8	1.136	0.000	0.000	9.091	3.214	1.136
			ATZ	100.00	8	0.000	0.000	0.000	0.000	0.000	0.000
		M	E2	0.20	8	6.250	0.000	0.000	50.000	17.678	6.250
			Ctrl	0.00	8	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	0.01	8	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	0.10	8	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	1.00	7	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	25.00	8	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	100.00	8	0.735	0.000	0.000	5.882	2.080	0.735

TABLE 2D.2. WILDLIFE EPL- Basic Summary Statistics for all Response Variables

No.	Response Variable (Grade>0 represents % in tank having any size effect; Grade>1 represents % in tank having effect with a severity level grade >1)	Sex	Chemical	Dose (ug/L)	No. of Tanks	Mean (%)	Median (%)	Min (%)	Max (%)	Standard Deviation	Standard Error
15	Other renal diagnoses (Grade>0)	F	E2	0.20	8	0.625	0.000	0.000	5.000	1.768	0.625
			Ctrl	0.00	8	3.314	0.000	0.000	9.091	4.580	1.619
			ATZ	0.01	8	0.893	0.000	0.000	7.143	2.525	0.893
			ATZ	0.10	8	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	1.00	7	2.092	0.000	0.000	9.091	3.716	1.405
			ATZ	25.00	8	6.635	0.000	0.000	30.000	10.979	3.882
			ATZ	100.00	8	0.000	0.000	0.000	0.000	0.000	0.000
		M	E2	0.20	8	2.083	0.000	0.000	16.667	5.893	2.083
			Ctrl	0.00	8	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	0.01	8	1.136	0.000	0.000	9.091	3.214	1.136
			ATZ	0.10	8	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	1.00	7	6.679	0.000	0.000	28.571	11.794	4.458
			ATZ	25.00	8	2.604	0.000	0.000	12.500	4.949	1.750
			ATZ	100.00	8	0.735	0.000	0.000	5.882	2.080	0.735
	Other renal diagnoses (Grade>1)	F	E2	0.20	8	0.625	0.000	0.000	5.000	1.768	0.625
			Ctrl	0.00	8	1.042	0.000	0.000	8.333	2.946	1.042
			ATZ	0.01	8	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	0.10	8	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	1.00	7	2.092	0.000	0.000	9.091	3.716	1.405
			ATZ	25.00	8	3.462	0.000	0.000	20.000	7.204	2.547
			ATZ	100.00	8	0.000	0.000	0.000	0.000	0.000	0.000
		M	E2	0.20	8	2.083	0.000	0.000	16.667	5.893	2.083

TABLE 2D.2. WILDLIFE EPL- Basic Summary Statistics for all Response Variables

No.	Response Variable (Grade>0 represents % in tank having any size effect; Grade>1 represents % in tank having effect with a severity level grade >1)	Sex	Chemical	Dose (ug/L)	No. of Tanks	Mean (%)	Median (%)	Min (%)	Max (%)	Standard Deviation	Standard Error
			Ctrl	0.00	8	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	0.01	8	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	0.10	8	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	1.00	7	2.041	0.000	0.000	14.286	5.399	2.041
			ATZ	25.00	8	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	100.00	8	0.735	0.000	0.000	5.882	2.080	0.735

TABLE 2D.3. IGB GROSS - Basic Summary Statistics for all Response Variables

No.	Response Variable (all except #s 3,4,5,6 are expressed as percents)	Sex	Chemical	Dose (ug/L)	No. of Tanks	Mean	Median	Min	Max	Standard Deviation	Standard Error
1	Tadpole mortality	None	E2	0.20	8	3.000	4.000	0.000	8.000	2.828	1.000
			-	0.00	16	2.260	2.000	0.000	8.000	2.525	0.631
			ATZ	0.01	8	4.000	2.000	0.000	16.000	5.657	2.000
			ATZ	0.10	8	2.000	0.000	0.000	8.000	3.024	1.069
			ATZ	1.00	8	2.500	2.000	0.000	8.000	2.976	1.052
			ATZ	25.00	8	2.500	4.000	0.000	4.000	2.070	0.732
			ATZ	100.00	8	3.000	2.000	0.000	12.000	4.140	1.464
2	Failure to complete metamorphosis	None	E2	0.20	8	0.000	0.000	0.000	0.000	0.000	0.000
			-	0.00	16	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	0.01	8	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	0.10	8	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	1.00	8	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	25.00	8	0.500	0.000	0.000	4.000	1.414	0.500
			ATZ	100.00	8	0.000	0.000	0.000	0.000	0.000	0.000
3	Age at completion of metamorphosis	F	E2	0.20	8	53.377	53.019	51.333	57.364	1.959	0.692
			-	0.00	16	51.664	51.896	48.722	54.556	1.782	0.446
			ATZ	0.01	8	50.423	50.648	47.714	52.733	1.688	0.597
			ATZ	0.10	8	51.919	52.092	50.188	53.429	1.057	0.374
			ATZ	1.00	8	50.945	51.344	48.733	53.091	1.363	0.482
			ATZ	25.00	8	51.775	51.538	50.444	53.692	1.161	0.411
			ATZ	100.00	8	51.606	51.547	49.000	54.154	1.643	0.581
		M	E2	0.20	8	54.785	54.167	52.500	57.500	1.629	0.576
			-	0.00	16	50.930	50.500	48.333	55.143	1.993	0.498
			ATZ	0.01	8	51.540	51.536	48.889	54.333	2.115	0.748
			ATZ	0.10	8	51.412	51.763	48.000	55.125	2.311	0.817

TABLE 2D.3. IGB GROSS - Basic Summary Statistics for all Response Variables

No.	Response Variable (all except #s 3,4,5,6 are expressed as percents)	Sex	Chemical	Dose (ug/L)	No. of Tanks	Mean	Median	Min	Max	Standard Deviation	Standard Error
			ATZ	1.00	8	51.457	50.756	48.923	57.111	2.516	0.889
			ATZ	25.00	8	50.468	49.874	48.545	52.900	1.671	0.591
			ATZ	100.00	8	51.063	51.231	49.000	52.813	1.383	0.489
4	Body weight	F	E2	0.20	8	0.562	0.557	0.520	0.617	0.033	0.012
			-	0.00	16	0.555	0.564	0.471	0.608	0.041	0.010
			ATZ	0.01	8	0.516	0.516	0.486	0.543	0.019	0.007
			ATZ	0.10	8	0.543	0.545	0.483	0.599	0.038	0.014
			ATZ	1.00	8	0.518	0.519	0.464	0.559	0.028	0.010
			ATZ	25.00	8	0.555	0.548	0.529	0.596	0.024	0.009
			ATZ	100.00	8	0.517	0.516	0.466	0.577	0.042	0.015
		M	E2	0.20	8	0.567	0.563	0.483	0.652	0.054	0.019
			-	0.00	16	0.539	0.540	0.445	0.600	0.040	0.010
			ATZ	0.01	8	0.522	0.525	0.479	0.560	0.026	0.009
			ATZ	0.10	8	0.519	0.512	0.464	0.590	0.040	0.014
			ATZ	1.00	8	0.514	0.506	0.451	0.575	0.045	0.016
			ATZ	25.00	8	0.517	0.505	0.486	0.585	0.033	0.012
			ATZ	100.00	8	0.522	0.536	0.418	0.587	0.054	0.019
5	Snout-to-vent length	F	E2	0.20	8	18.560	18.676	17.923	19.156	0.408	0.144
			-	0.00	16	18.351	18.357	17.500	19.400	0.471	0.118
			ATZ	0.01	8	17.904	17.929	17.429	18.143	0.235	0.083
			ATZ	0.10	8	18.207	18.160	17.833	18.700	0.318	0.113
			ATZ	1.00	8	17.959	17.909	17.563	18.444	0.282	0.100
			ATZ	25.00	8	18.284	18.252	18.000	18.615	0.206	0.073
			ATZ	100.00	8	17.848	17.716	17.125	18.667	0.533	0.188
		M	E2	0.20	8	18.376	18.450	17.000	19.500	0.771	0.273
			-	0.00	16	18.144	18.133	17.444	18.750	0.356	0.089

TABLE 2D.3. IGB GROSS - Basic Summary Statistics for all Response Variables

No.	Response Variable (all except #s 3,4,5,6 are expressed as percents)	Sex	Chemical	Dose (ug/L)	No. of Tanks	Mean	Median	Min	Max	Standard Deviation	Standard Error
			ATZ	0.01	8	18.046	18.101	17.667	18.286	0.231	0.082
			ATZ	0.10	8	18.028	18.063	17.400	18.769	0.481	0.170
			ATZ	1.00	8	17.809	17.821	17.308	18.278	0.350	0.124
			ATZ	25.00	8	17.831	17.814	17.308	18.500	0.346	0.122
			ATZ	100.00	8	17.959	18.160	17.063	18.467	0.474	0.168
6	Gonadal image area	F	E2	0.20	8	1.653	1.619	1.458	2.015	0.182	0.064
			-	0.00	16	1.675	1.668	1.363	1.935	0.156	0.039
			ATZ	0.01	8	1.534	1.542	1.373	1.749	0.130	0.046
			ATZ	0.10	8	1.611	1.608	1.425	1.754	0.110	0.039
			ATZ	1.00	8	1.606	1.595	1.468	1.799	0.101	0.036
			ATZ	25.00	8	1.602	1.620	1.458	1.767	0.103	0.036
			ATZ	100.00	8	1.571	1.596	1.395	1.652	0.082	0.029
		M	E2	0.20	8	1.076	1.087	0.694	1.435	0.211	0.075
			-	0.00	16	0.916	0.915	0.784	1.010	0.064	0.016
			ATZ	0.01	8	0.898	0.903	0.790	0.974	0.058	0.020
			ATZ	0.10	8	0.856	0.837	0.793	1.004	0.067	0.024
			ATZ	1.00	8	0.854	0.859	0.765	0.916	0.053	0.019
			ATZ	25.00	8	0.872	0.887	0.771	0.949	0.064	0.023
			ATZ	100.00	8	0.842	0.857	0.712	0.933	0.079	0.028
7	Intersex (gross observation)	None	E2	0.20	8	0.000	0.000	0.000	0.000	0.000	0.000
			-	0.00	16	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	0.01	8	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	0.10	8	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	1.00	8	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	25.00	8	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	100.00	8	0.000	0.000	0.000	0.000	0.000	0.000

TABLE 2D.3. IGB GROSS - Basic Summary Statistics for all Response Variables

No.	Response Variable (all except #s 3,4,5,6 are expressed as percents)	Sex	Chemical	Dose (ug/L)	No. of Tanks	Mean	Median	Min	Max	Standard Deviation	Standard Error
8	Mixed tissue types (gross observation)	None	E2	0.20	8	4.106	4.000	0.000	12.500	4.412	1.560
			-	0.00	16	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	0.01	8	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	0.10	8	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	1.00	8	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	25.00	8	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	100.00	8	0.000	0.000	0.000	0.000	0.000	0.000
9	Gonadal adhesions	F	E2	0.20	8	0.000	0.000	0.000	0.000	0.000	0.000
			-	0.00	16	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	0.01	8	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	0.10	8	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	1.00	8	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	25.00	8	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	100.00	8	0.000	0.000	0.000	0.000	0.000	0.000
		M	E2	0.20	8	0.000	0.000	0.000	0.000	0.000	0.000
			-	0.00	16	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	0.01	8	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	0.10	8	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	1.00	8	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	25.00	8	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	100.00	8	0.000	0.000	0.000	0.000	0.000	0.000
10	Gonadal aplasia	F	E2	0.20	8	0.000	0.000	0.000	0.000	0.000	0.000
			-	0.00	16	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	0.01	8	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	0.10	8	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	1.00	8	0.000	0.000	0.000	0.000	0.000	0.000

TABLE 2D.3. IGB GROSS - Basic Summary Statistics for all Response Variables

No.	Response Variable (all except #s 3,4,5,6 are expressed as percents)	Sex	Chemical	Dose (ug/L)	No. of Tanks	Mean	Median	Min	Max	Standard Deviation	Standard Error
			ATZ	25.00	8	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	100.00	8	0.000	0.000	0.000	0.000	0.000	0.000
		M	E2	0.20	8	0.000	0.000	0.000	0.000	0.000	0.000
			-	0.00	16	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	0.01	8	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	0.10	8	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	1.00	8	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	25.00	8	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	100.00	8	0.000	0.000	0.000	0.000	0.000	0.000
11	Gonadal segmental aplasia	F	E2	0.20	8	0.694	0.000	0.000	5.556	1.964	0.694
			-	0.00	16	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	0.01	8	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	0.10	8	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	1.00	8	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	25.00	8	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	100.00	8	1.389	0.000	0.000	11.111	3.928	1.389
		M	E2	0.20	8	27.083	29.167	0.000	50.000	19.288	6.819
			-	0.00	16	2.055	0.000	0.000	11.765	4.430	1.107
			ATZ	0.01	8	1.823	0.000	0.000	8.333	3.421	1.210
			ATZ	0.10	8	2.976	0.000	0.000	10.000	4.219	1.492
			ATZ	1.00	8	1.389	0.000	0.000	11.111	3.928	1.389
			ATZ	25.00	8	1.875	0.000	0.000	8.333	3.500	1.238
			ATZ	100.00	8	4.920	3.125	0.000	18.750	6.553	2.317
12	Gonadal bifurcation	F	E2	0.20	8	0.000	0.000	0.000	0.000	0.000	0.000
			-	0.00	16	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	0.01	8	0.000	0.000	0.000	0.000	0.000	0.000

TABLE 2D.3. IGB GROSS - Basic Summary Statistics for all Response Variables

No.	Response Variable (all except #s 3,4,5,6 are expressed as percents)	Sex	Chemical	Dose (ug/L)	No. of Tanks	Mean	Median	Min	Max	Standard Deviation	Standard Error
			ATZ	0.10	8	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	1.00	8	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	25.00	8	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	100.00	8	0.000	0.000	0.000	0.000	0.000	0.000
		M	E2	0.20	8	0.000	0.000	0.000	0.000	0.000	0.000
			-	0.00	16	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	0.01	8	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	0.10	8	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	1.00	8	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	25.00	8	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	100.00	8	0.000	0.000	0.000	0.000	0.000	0.000
13	Gonadal deformity	F	E2	0.20	8	3.182	0.000	0.000	12.500	4.813	1.701
			-	0.00	16	7.304	7.143	0.000	21.429	6.536	1.634
			ATZ	0.01	8	8.610	8.889	0.000	21.429	8.236	2.912
			ATZ	0.10	8	4.253	3.125	0.000	11.111	4.814	1.702
			ATZ	1.00	8	5.650	4.167	0.000	16.667	6.524	2.307
			ATZ	25.00	8	7.418	3.846	0.000	28.571	10.220	3.613
			ATZ	100.00	8	6.922	5.556	0.000	20.000	7.881	2.786
		M	E2	0.20	8	0.000	0.000	0.000	0.000	0.000	0.000
			-	0.00	16	0.568	0.000	0.000	9.091	2.273	0.568
			ATZ	0.01	8	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	0.10	8	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	1.00	8	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	25.00	8	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	100.00	8	0.000	0.000	0.000	0.000	0.000	0.000
14	Gonadal displacement	F	E2	0.20	8	1.439	0.000	0.000	6.250	2.678	0.947

TABLE 2D.3. IGB GROSS - Basic Summary Statistics for all Response Variables

No.	Response Variable (all except #s 3,4,5,6 are expressed as percents)	Sex	Chemical	Dose (ug/L)	No. of Tanks	Mean	Median	Min	Max	Standard Deviation	Standard Error
			-	0.00	16	2.207	0.000	0.000	8.333	3.420	0.855
			ATZ	0.01	8	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	0.10	8	1.563	0.000	0.000	6.250	2.893	1.023
			ATZ	1.00	8	3.003	0.000	0.000	11.111	4.388	1.551
			ATZ	25.00	8	2.679	0.000	0.000	14.286	5.314	1.879
			ATZ	100.00	8	0.735	0.000	0.000	5.882	2.080	0.735
		M	E2	0.20	8	3.889	0.000	0.000	20.000	7.583	2.681
			-	0.00	16	2.848	0.000	0.000	9.091	3.871	0.968
			ATZ	0.01	8	5.787	3.125	0.000	16.667	6.925	2.448
			ATZ	0.10	8	5.839	4.545	0.000	14.286	6.456	2.283
			ATZ	1.00	8	4.077	3.333	0.000	11.111	4.554	1.610
			ATZ	25.00	8	4.068	0.000	0.000	18.182	6.566	2.322
			ATZ	100.00	8	4.688	3.333	0.000	12.500	5.269	1.863
15	Fused gonads	F	E2	0.20	8	0.658	0.000	0.000	5.263	1.861	0.658
			-	0.00	16	0.927	0.000	0.000	7.692	2.536	0.634
			ATZ	0.01	8	0.833	0.000	0.000	6.667	2.357	0.833
			ATZ	0.10	8	0.833	0.000	0.000	6.667	2.357	0.833
			ATZ	1.00	8	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	25.00	8	1.854	0.000	0.000	7.692	3.437	1.215
			ATZ	100.00	8	2.298	0.000	0.000	12.500	4.608	1.629
		M	E2	0.20	8	0.000	0.000	0.000	0.000	0.000	0.000
			-	0.00	16	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	0.01	8	0.781	0.000	0.000	6.250	2.210	0.781
			ATZ	0.10	8	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	1.00	8	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	25.00	8	0.000	0.000	0.000	0.000	0.000	0.000

TABLE 2D.3. IGB GROSS - Basic Summary Statistics for all Response Variables

No.	Response Variable (all except #s 3,4,5,6 are expressed as percents)	Sex	Chemical	Dose (ug/L)	No. of Tanks	Mean	Median	Min	Max	Standard Deviation	Standard Error
			ATZ	100.00	8	2.003	0.000	0.000	8.333	3.713	1.313
16	Gonadal hypertrophy	F	E2	0.20	8	29.772	26.136	18.182	46.154	11.056	3.909
			-	0.00	16	35.311	33.482	7.143	58.333	13.624	3.406
			ATZ	0.01	8	29.112	29.670	6.250	44.444	12.626	4.464
			ATZ	0.10	8	32.061	30.952	0.000	62.500	19.291	6.820
			ATZ	1.00	8	33.952	30.303	16.667	55.556	12.737	4.503
			ATZ	25.00	8	36.909	34.524	23.077	50.000	9.341	3.303
			ATZ	100.00	8	26.288	26.923	0.000	60.000	20.074	7.097
		M	E2	0.20	8	35.972	36.111	0.000	83.333	29.713	10.505
			-	0.00	16	31.748	28.636	0.000	77.778	17.594	4.398
			ATZ	0.01	8	35.075	32.292	18.182	57.143	13.055	4.616
			ATZ	0.10	8	19.492	21.825	0.000	30.769	9.401	3.324
			ATZ	1.00	8	24.155	24.747	0.000	46.154	14.371	5.081
			ATZ	25.00	8	25.233	20.629	9.091	60.000	15.453	5.463
			ATZ	100.00	8	18.898	17.424	10.000	31.250	6.879	2.432
17	Gonadal segmental hypertrophy	F	E2	0.20	8	0.000	0.000	0.000	0.000	0.000	0.000
			-	0.00	16	0.794	0.000	0.000	7.143	2.188	0.547
			ATZ	0.01	8	1.743	0.000	0.000	7.692	3.250	1.149
			ATZ	0.10	8	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	1.00	8	1.136	0.000	0.000	9.091	3.214	1.136
			ATZ	25.00	8	4.739	3.571	0.000	15.385	5.687	2.011
			ATZ	100.00	8	0.833	0.000	0.000	6.667	2.357	0.833
		M	E2	0.20	8	0.000	0.000	0.000	0.000	0.000	0.000
			-	0.00	16	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	0.01	8	0.893	0.000	0.000	7.143	2.525	0.893
			ATZ	0.10	8	0.000	0.000	0.000	0.000	0.000	0.000

TABLE 2D.3. IGB GROSS - Basic Summary Statistics for all Response Variables

No.	Response Variable (all except #s 3,4,5,6 are expressed as percents)	Sex	Chemical	Dose (ug/L)	No. of Tanks	Mean	Median	Min	Max	Standard Deviation	Standard Error
			ATZ	1.00	8	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	25.00	8	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	100.00	8	1.136	0.000	0.000	9.091	3.214	1.136
18	Gonadal hypoplasia	F	E2	0.20	8	49.618	48.077	36.842	63.636	8.911	3.151
			-	0.00	16	37.917	37.088	20.000	57.143	9.577	2.394
			ATZ	0.01	8	49.122	48.333	23.077	85.714	18.436	6.518
			ATZ	0.10	8	30.337	31.250	6.667	57.143	16.415	5.804
			ATZ	1.00	8	35.983	33.333	22.222	53.333	10.175	3.597
			ATZ	25.00	8	39.513	34.524	23.077	69.231	15.347	5.426
			ATZ	100.00	8	31.527	30.385	11.111	50.000	12.532	4.431
		M	E2	0.20	8	2.778	0.000	0.000	11.111	5.143	1.818
			-	0.00	16	5.700	3.333	0.000	27.273	7.479	1.870
			ATZ	0.01	8	9.146	7.292	0.000	22.222	9.214	3.258
			ATZ	0.10	8	6.705	7.879	0.000	15.385	6.098	2.156
			ATZ	1.00	8	5.107	3.333	0.000	15.385	6.037	2.134
			ATZ	25.00	8	10.029	8.333	0.000	30.000	10.737	3.796
			ATZ	100.00	8	5.744	6.250	0.000	16.667	5.779	2.043
19	Gonadal segmental hypoplasia	F	E2	0.20	8	7.024	7.670	0.000	15.385	5.312	1.878
			-	0.00	16	3.228	0.000	0.000	22.222	6.032	1.508
			ATZ	0.01	8	3.281	0.000	0.000	13.333	5.002	1.769
			ATZ	0.10	8	8.063	7.738	0.000	18.750	6.280	2.220
			ATZ	1.00	8	2.656	0.000	0.000	8.333	3.713	1.313
			ATZ	25.00	8	8.051	7.692	0.000	15.385	5.948	2.103
			ATZ	100.00	8	7.033	2.941	0.000	25.000	9.262	3.275
		M	E2	0.20	8	0.000	0.000	0.000	0.000	0.000	0.000
			-	0.00	16	1.928	0.000	0.000	11.765	4.176	1.044

TABLE 2D.3. IGB GROSS - Basic Summary Statistics for all Response Variables

No.	Response Variable (all except #s 3,4,5,6 are expressed as percents)	Sex	Chemical	Dose (ug/L)	No. of Tanks	Mean	Median	Min	Max	Standard Deviation	Standard Error
			ATZ	0.01	8	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	0.10	8	3.115	0.000	0.000	11.111	4.493	1.588
			ATZ	1.00	8	1.136	0.000	0.000	9.091	3.214	1.136
			ATZ	25.00	8	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	100.00	8	2.178	0.000	0.000	9.091	4.038	1.428
20	Gonadal translucence	F	E2	0.20	8	0.962	0.000	0.000	7.692	2.720	0.962
			-	0.00	16	1.766	0.000	0.000	11.111	3.869	0.967
			ATZ	0.01	8	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	0.10	8	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	1.00	8	1.042	0.000	0.000	8.333	2.946	1.042
			ATZ	25.00	8	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	100.00	8	0.000	0.000	0.000	0.000	0.000	0.000
		M	E2	0.20	8	54.375	50.000	44.444	75.000	9.514	3.364
			-	0.00	16	10.648	9.091	0.000	42.857	11.745	2.936
			ATZ	0.01	8	11.375	14.583	0.000	22.222	9.874	3.491
			ATZ	0.10	8	16.469	13.942	0.000	37.500	14.401	5.092
			ATZ	1.00	8	10.508	11.111	0.000	23.077	7.742	2.737
			ATZ	25.00	8	20.086	19.091	6.667	36.364	10.100	3.571
			ATZ	100.00	8	13.279	6.971	0.000	42.857	14.731	5.208
21	Gonadal segmental translucence	F	E2	0.20	8	0.962	0.000	0.000	7.692	2.720	0.962
			-	0.00	16	0.694	0.000	0.000	11.111	2.778	0.694
			ATZ	0.01	8	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	0.10	8	1.250	0.000	0.000	10.000	3.536	1.250
			ATZ	1.00	8	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	25.00	8	1.136	0.000	0.000	9.091	3.214	1.136
			ATZ	100.00	8	0.000	0.000	0.000	0.000	0.000	0.000

TABLE 2D.3. IGB GROSS - Basic Summary Statistics for all Response Variables

No.	Response Variable (all except #s 3,4,5,6 are expressed as percents)	Sex	Chemical	Dose (ug/L)	No. of Tanks	Mean	Median	Min	Max	Standard Deviation	Standard Error
		M	E2	0.20	8	15.764	5.556	0.000	50.000	20.215	7.147
			-	0.00	16	7.724	8.013	0.000	20.000	7.355	1.839
			ATZ	0.01	8	2.865	0.000	0.000	16.667	5.990	2.118
			ATZ	0.10	8	7.644	7.879	0.000	20.000	7.437	2.629
			ATZ	1.00	8	6.209	3.846	0.000	20.000	7.691	2.719
			ATZ	25.00	8	6.246	7.179	0.000	18.182	6.259	2.213
			ATZ	100.00	8	3.793	3.125	0.000	9.091	4.149	1.467
22	Gonadal pearling	F	E2	0.20	8	0.000	0.000	0.000	0.000	0.000	0.000
			-	0.00	16	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	0.01	8	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	0.10	8	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	1.00	8	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	25.00	8	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	100.00	8	0.000	0.000	0.000	0.000	0.000	0.000
		M	E2	0.20	8	0.000	0.000	0.000	0.000	0.000	0.000
			-	0.00	16	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	0.01	8	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	0.10	8	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	1.00	8	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	25.00	8	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	100.00	8	0.000	0.000	0.000	0.000	0.000	0.000
23	Melanophores decreased in ovaries	F	E2	0.20	8	10.255	7.292	0.000	30.769	9.380	3.316
			-	0.00	16	7.826	7.143	0.000	21.429	7.099	1.775
			ATZ	0.01	8	8.063	6.905	0.000	23.077	8.364	2.957
			ATZ	0.10	8	8.931	6.905	0.000	22.222	7.785	2.753
			ATZ	1.00	8	8.515	8.333	0.000	25.000	7.829	2.768

TABLE 2D.3. IGB GROSS - Basic Summary Statistics for all Response Variables

No.	Response Variable (all except #s 3,4,5,6 are expressed as percents)	Sex	Chemical	Dose (ug/L)	No. of Tanks	Mean	Median	Min	Max	Standard Deviation	Standard Error
			ATZ	25.00	8	13.858	12.698	7.143	30.769	7.599	2.687
			ATZ	100.00	8	14.550	12.132	0.000	33.333	9.962	3.522
24	Internal melanophores in testes	M	E2	0.20	8	0.000	0.000	0.000	0.000	0.000	0.000
			-	0.00	16	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	0.01	8	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	0.10	8	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	1.00	8	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	25.00	8	0.833	0.000	0.000	6.667	2.357	0.833
			ATZ	100.00	8	0.000	0.000	0.000	0.000	0.000	0.000
25	Frequency of males among just males and females	None	E2	0.20	8	27.160	26.970	9.524	45.000	12.001	4.243
			-	0.00	16	47.199	44.000	28.000	70.833	11.134	2.783
			ATZ	0.01	8	46.513	44.000	30.435	66.667	12.805	4.527
			ATZ	0.10	8	44.942	42.000	33.333	60.870	11.371	4.020
			ATZ	1.00	8	49.749	52.000	36.000	62.500	9.056	3.202
			ATZ	25.00	8	48.188	45.833	41.667	62.500	6.993	2.472
			ATZ	100.00	8	51.595	53.545	29.167	66.667	13.046	4.613
26	Frequency of males	None	E2	0.20	8	22.174	18.696	8.333	37.500	10.425	3.686
			-	0.00	16	47.199	44.000	28.000	70.833	11.134	2.783
			ATZ	0.01	8	46.513	44.000	30.435	66.667	12.805	4.527
			ATZ	0.10	8	44.942	42.000	33.333	60.870	11.371	4.020
			ATZ	1.00	8	49.749	52.000	36.000	62.500	9.056	3.202
			ATZ	25.00	8	47.917	45.833	41.667	62.500	6.767	2.392
			ATZ	100.00	8	51.595	53.545	29.167	66.667	13.046	4.613
27	Frequency of females	None	E2	0.20	8	59.728	58.000	45.833	79.167	12.743	4.505
			-	0.00	16	52.801	56.000	29.167	72.000	11.134	2.783

TABLE 2D.3. IGB GROSS - Basic Summary Statistics for all Response Variables

No.	Response Variable (all except #s 3,4,5,6 are expressed as percents)	Sex	Chemical	Dose (ug/L)	No. of Tanks	Mean	Median	Min	Max	Standard Deviation	Standard Error
			ATZ	0.01	8	53.487	56.000	33.333	69.565	12.805	4.527
			ATZ	0.10	8	55.058	58.000	39.130	66.667	11.371	4.020
			ATZ	1.00	8	50.251	48.000	37.500	64.000	9.056	3.202
			ATZ	25.00	8	51.583	54.167	37.500	58.333	7.242	2.561
			ATZ	100.00	8	48.405	46.455	33.333	70.833	13.046	4.613
28	Frequency of intersex	None	E2	0.20	8	0.000	0.000	0.000	0.000	0.000	0.000
			-	0.00	16	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	0.01	8	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	0.10	8	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	1.00	8	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	25.00	8	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	100.00	8	0.000	0.000	0.000	0.000	0.000	0.000
29	Frequency of mixed sex	None	E2	0.20	8	18.098	14.583	8.333	34.783	9.081	3.211
			-	0.00	16	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	0.01	8	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	0.10	8	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	1.00	8	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	25.00	8	0.500	0.000	0.000	4.000	1.414	0.500
			ATZ	100.00	8	0.000	0.000	0.000	0.000	0.000	0.000

Table 2D.4. IGB EPL - Basic Summary Statistics for all Response Variables

No.	Response Variable (Grade>0 represents % in tank having any size effect; Grade>1 represents % in tank having effect with a severity level grade >1)	Sex	Chemical	Dose (ug/L)	No. of Tanks	Mean (%)	Median (%)	Min (%)	Max (%)	Standard Deviation	Standard Error
1	Segmental hypoplasia (Grade>0)	F	E2	0.20	8	5.013	5.903	0.000	9.091	3.327	1.176
			Ctl	0.00	16	1.453	0.000	0.000	10.000	3.228	0.807
			ATZ	0.01	8	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	0.10	8	1.563	0.000	0.000	12.500	4.419	1.563
			ATZ	1.00	8	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	25.00	8	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	100.00	8	1.389	0.000	0.000	11.111	3.928	1.389
		M	E2	0.20	8	25.347	29.167	0.000	50.000	22.588	7.986
			Ctl	0.00	16	8.006	9.091	0.000	18.182	6.279	1.570
			ATZ	0.01	8	9.874	12.698	0.000	18.750	8.514	3.010
			ATZ	0.10	8	14.191	12.917	0.000	27.273	7.949	2.810
			ATZ	1.00	8	8.280	7.179	0.000	30.769	10.180	3.599
			ATZ	25.00	8	9.034	4.545	0.000	25.000	10.592	3.745
			ATZ	100.00	8	10.651	13.393	0.000	18.750	7.022	2.483
	Segmental hypoplasia (Grade>1)	F	E2	0.20	8	0.000	0.000	0.000	0.000	0.000	0.000
			Ctl	0.00	16	0.481	0.000	0.000	7.692	1.923	0.481
			ATZ	0.01	8	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	0.10	8	0.000	0.000	0.000	0.000	0.000	0.000

Table 2D.4. IGB EPL - Basic Summary Statistics for all Response Variables

No.	Response Variable (Grade>0 represents % in tank having any size effect; Grade>1 represents % in tank having effect with a severity level grade >1)	Sex	Chemical	Dose (ug/L)	No. of Tanks	Mean (%)	Median (%)	Min (%)	Max (%)	Standard Deviation	Standard Error
			ATZ	1.00	8	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	25.00	8	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	100.00	8	1.389	0.000	0.000	11.111	3.928	1.389
		M	E2	0.20	8	7.292	0.000	0.000	25.000	10.803	3.819
			Ctl	0.00	16	0.784	0.000	0.000	6.667	2.148	0.537
			ATZ	0.01	8	1.136	0.000	0.000	9.091	3.214	1.136
			ATZ	0.10	8	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	1.00	8	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	25.00	8	1.136	0.000	0.000	9.091	3.214	1.136
			ATZ	100.00	8	0.781	0.000	0.000	6.250	2.210	0.781
2	Segmental aplasia (Grade>0)	F	E2	0.20	8	0.694	0.000	0.000	5.556	1.964	0.694
			Ctl	0.00	16	0.391	0.000	0.000	6.250	1.563	0.391
			ATZ	0.01	8	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	0.10	8	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	1.00	8	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	25.00	8	1.136	0.000	0.000	9.091	3.214	1.136
			ATZ	100.00	8	0.000	0.000	0.000	0.000	0.000	0.000
		M	E2	0.20	8	3.125	0.000	0.000	25.000	8.839	3.125

Table 2D.4. IGB EPL - Basic Summary Statistics for all Response Variables

No.	Response Variable (Grade>0 represents % in tank having any size effect; Grade>1 represents % in tank having effect with a severity level grade >1)	Sex	Chemical	Dose (ug/L)	No. of Tanks	Mean (%)	Median (%)	Min (%)	Max (%)	Standard Deviation	Standard Error
			Ctl	0.00	16	3.548	0.000	0.000	18.182	5.922	1.481
			ATZ	0.01	8	3.418	0.000	0.000	11.111	4.835	1.710
			ATZ	0.10	8	2.143	0.000	0.000	10.000	4.041	1.429
			ATZ	1.00	8	3.312	0.000	0.000	11.111	4.691	1.659
			ATZ	25.00	8	8.746	8.712	0.000	18.182	4.991	1.765
			ATZ	100.00	8	6.914	6.971	0.000	18.750	6.979	2.467
	Segmental aplasia (Grade>1)	F	E2	0.20	8	0.694	0.000	0.000	5.556	1.964	0.694
			Ctl	0.00	16	0.391	0.000	0.000	6.250	1.563	0.391
			ATZ	0.01	8	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	0.10	8	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	1.00	8	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	25.00	8	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	100.00	8	0.000	0.000	0.000	0.000	0.000	0.000
		M	E2	0.20	8	0.000	0.000	0.000	0.000	0.000	0.000
			Ctl	0.00	16	0.568	0.000	0.000	9.091	2.273	0.568
			ATZ	0.01	8	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	0.10	8	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	1.00	8	1.389	0.000	0.000	11.111	3.928	1.389

Table 2D.4. IGB EPL - Basic Summary Statistics for all Response Variables

No.	Response Variable (Grade>0 represents % in tank having any size effect; Grade>1 represents % in tank having effect with a severity level grade >1)	Sex	Chemical	Dose (ug/L)	No. of Tanks	Mean (%)	Median (%)	Min (%)	Max (%)	Standard Deviation	Standard Error
			ATZ	25.00	8	0.833	0.000	0.000	6.667	2.357	0.833
			ATZ	100.00	8	2.784	0.000	0.000	8.333	3.885	1.374
3	Decreased germ cells in gonad (Grade>0)	F	E2	0.20	8	41.958	41.148	30.769	55.556	10.697	3.782
			Ctl	0.00	16	41.989	43.304	14.286	66.667	13.322	3.331
			ATZ	0.01	8	47.204	44.762	33.333	64.286	10.908	3.857
			ATZ	0.10	8	52.587	48.333	31.250	77.778	17.396	6.150
			ATZ	1.00	8	42.457	44.602	33.333	58.333	8.776	3.103
			ATZ	25.00	8	39.328	40.659	23.077	46.154	7.573	2.677
			ATZ	100.00	8	48.024	50.000	22.222	62.500	12.650	4.472
		M	E2	0.20	8	72.153	70.833	50.000	100.000	17.594	6.221
			Ctl	0.00	16	70.079	71.008	45.455	90.909	12.118	3.029
			ATZ	0.01	8	70.141	69.048	42.857	88.889	15.716	5.557
			ATZ	0.10	8	70.605	76.667	46.154	88.889	17.092	6.043
			ATZ	1.00	8	71.961	71.667	46.154	100.000	16.473	5.824
			ATZ	25.00	8	69.436	68.333	50.000	92.308	13.255	4.686
			ATZ	100.00	8	71.002	73.214	54.545	87.500	11.217	3.966
	Decreased germ cells in gonad (Grade>1)	F	E2	0.20	8	30.577	28.289	18.182	50.000	11.437	4.044
			Ctl	0.00	16	26.035	25.824	8.333	44.444	9.984	2.496

Table 2D.4. IGB EPL - Basic Summary Statistics for all Response Variables

No.	Response Variable (Grade>0 represents % in tank having any size effect; Grade>1 represents % in tank having effect with a severity level grade >1)	Sex	Chemical	Dose (ug/L)	No. of Tanks	Mean (%)	Median (%)	Min (%)	Max (%)	Standard Deviation	Standard Error
			ATZ	0.01	8	36.310	40.000	21.429	57.143	12.842	4.540
			ATZ	0.10	8	29.167	29.167	10.000	50.000	16.661	5.890
			ATZ	1.00	8	24.961	27.273	0.000	41.667	12.529	4.430
			ATZ	25.00	8	29.073	29.021	15.385	42.857	9.135	3.230
			ATZ	100.00	8	27.405	27.206	15.385	41.667	8.047	2.845
		M	E2	0.20	8	57.083	52.778	33.333	77.778	17.025	6.019
			Ctl	0.00	16	44.033	40.064	26.667	70.000	13.235	3.309
			ATZ	0.01	8	46.665	48.701	18.182	66.667	18.685	6.606
			ATZ	0.10	8	40.853	36.039	23.077	70.000	15.854	5.605
			ATZ	1.00	8	46.703	43.651	23.077	69.231	14.410	5.095
			ATZ	25.00	8	43.199	42.727	16.667	69.231	15.657	5.536
			ATZ	100.00	8	34.171	31.667	14.286	50.000	11.822	4.180
4	Dilated testis tubules (Grade>0)	M	E2	0.20	8	63.056	57.778	33.333	100.000	20.092	7.104
			Ctl	0.00	16	10.749	7.879	0.000	44.444	13.002	3.251
			ATZ	0.01	8	11.085	8.712	0.000	28.571	8.697	3.075
			ATZ	0.10	8	13.518	12.500	0.000	27.273	8.060	2.850
			ATZ	1.00	8	9.988	8.846	0.000	35.714	11.933	4.219
			ATZ	25.00	8	18.036	19.091	0.000	33.333	10.529	3.723

Table 2D.4. IGB EPL - Basic Summary Statistics for all Response Variables

No.	Response Variable (Grade>0 represents % in tank having any size effect; Grade>1 represents % in tank having effect with a severity level grade >1)	Sex	Chemical	Dose (ug/L)	No. of Tanks	Mean (%)	Median (%)	Min (%)	Max (%)	Standard Deviation	Standard Error
			ATZ	100.00	8	11.155	9.545	6.250	18.750	4.458	1.576
	Dilated testis tubules (Grade>1)	M	E2	0.20	8	47.431	52.778	0.000	75.000	22.398	7.919
			Ctl	0.00	16	2.493	0.000	0.000	11.111	3.972	0.993
			ATZ	0.01	8	1.674	0.000	0.000	7.143	3.109	1.099
			ATZ	0.10	8	3.125	0.000	0.000	12.500	5.786	2.046
			ATZ	1.00	8	3.600	0.000	0.000	11.111	5.056	1.787
			ATZ	25.00	8	4.181	0.000	0.000	16.667	6.322	2.235
			ATZ	100.00	8	3.497	3.125	0.000	8.333	3.794	1.341
5	Dividing gonocytes in testes (Grade>0)	M	E2	0.20	8	45.278	45.000	0.000	77.778	28.012	9.904
			Ctl	0.00	16	16.212	18.182	0.000	33.333	9.636	2.409
			ATZ	0.01	8	22.671	20.202	0.000	58.333	17.054	6.030
			ATZ	0.10	8	16.345	21.111	0.000	28.571	11.958	4.228
			ATZ	1.00	8	26.775	24.872	10.000	46.154	12.112	4.282
			ATZ	25.00	8	21.591	19.091	0.000	36.364	11.607	4.104
			ATZ	100.00	8	15.018	16.234	0.000	25.000	7.767	2.746
	Dividing gonocytes in testes (Grade>1)	M	E2	0.20	8	12.153	0.000	0.000	50.000	18.660	6.597
			Ctl	0.00	16	1.111	0.000	0.000	11.111	3.143	0.786

Table 2D.4. IGB EPL - Basic Summary Statistics for all Response Variables

No.	Response Variable (Grade>0 represents % in tank having any size effect; Grade>1 represents % in tank having effect with a severity level grade >1)	Sex	Chemical	Dose (ug/L)	No. of Tanks	Mean (%)	Median (%)	Min (%)	Max (%)	Standard Deviation	Standard Error
			ATZ	0.01	8	2.178	0.000	0.000	9.091	4.038	1.428
			ATZ	0.10	8	2.922	0.000	0.000	14.286	5.586	1.975
			ATZ	1.00	8	4.167	0.000	0.000	22.222	8.267	2.923
			ATZ	25.00	8	5.492	3.333	0.000	18.182	6.722	2.377
			ATZ	100.00	8	0.000	0.000	0.000	0.000	0.000	0.000
6	Internal melanophores in testis (Grade>0)	M	E2	0.20	8	77.639	75.000	60.000	100.000	14.957	5.288
			Ctl	0.00	16	58.502	63.068	11.111	81.818	18.031	4.508
			ATZ	0.01	8	59.110	60.714	27.273	81.818	18.537	6.554
			ATZ	0.10	8	66.337	63.393	46.667	88.889	14.731	5.208
			ATZ	1.00	8	58.352	64.615	20.000	76.923	18.622	6.584
			ATZ	25.00	8	64.104	65.152	40.000	80.000	13.114	4.636
			ATZ	100.00	8	48.753	48.798	20.000	72.727	15.439	5.458
	Internal melanophores in testis (Grade>1)	M	E2	0.20	8	41.806	42.222	0.000	100.000	29.234	10.336
			Ctl	0.00	16	12.655	12.692	0.000	29.412	11.132	2.783
			ATZ	0.01	8	16.562	16.518	8.333	27.273	6.904	2.441
			ATZ	0.10	8	15.183	14.835	0.000	30.000	8.510	3.009
			ATZ	1.00	8	8.777	7.692	0.000	22.222	6.255	2.212
			ATZ	25.00	8	7.946	9.091	0.000	16.667	7.096	2.509

Table 2D.4. IGB EPL - Basic Summary Statistics for all Response Variables

No.	Response Variable (Grade>0 represents % in tank having any size effect; Grade>1 represents % in tank having effect with a severity level grade >1)	Sex	Chemical	Dose (ug/L)	No. of Tanks	Mean (%)	Median (%)	Min (%)	Max (%)	Standard Deviation	Standard Error
			ATZ	100.00	8	3.921	3.125	0.000	9.091	4.265	1.508
7	Decreased ovarian pigmentation (Grade>0)	F	E2	0.20	8	6.936	5.903	0.000	23.077	7.241	2.560
			Ctl	0.00	16	6.963	7.143	0.000	15.385	5.674	1.418
			ATZ	0.01	8	7.170	6.905	0.000	23.077	7.978	2.821
			ATZ	0.10	8	7.889	6.905	0.000	22.222	7.133	2.522
			ATZ	1.00	8	8.515	8.333	0.000	25.000	7.829	2.768
			ATZ	25.00	8	11.760	9.402	0.000	30.769	9.055	3.202
			ATZ	100.00	8	13.161	12.132	0.000	22.222	7.420	2.623
	Decreased ovarian pigmentation (Grade>1)	F	E2	0.20	8	1.918	0.000	0.000	9.091	3.631	1.284
			Ctl	0.00	16	3.030	0.000	0.000	7.692	3.574	0.894
			ATZ	0.01	8	3.452	3.333	0.000	7.143	3.695	1.306
			ATZ	0.10	8	5.459	6.458	0.000	12.500	5.012	1.772
			ATZ	1.00	8	8.515	8.333	0.000	25.000	7.829	2.768
			ATZ	25.00	8	5.166	7.418	0.000	11.111	4.447	1.572
			ATZ	100.00	8	9.561	10.882	0.000	22.222	8.922	3.154
8	Decreased ovarian cavity size (Grade>0)	F	E2	0.20	8	9.014	8.392	0.000	18.750	6.122	2.165
			Ctl	0.00	16	6.806	7.143	0.000	22.222	6.474	1.618

Table 2D.4. IGB EPL - Basic Summary Statistics for all Response Variables

No.	Response Variable (Grade>0 represents % in tank having any size effect; Grade>1 represents % in tank having effect with a severity level grade >1)	Sex	Chemical	Dose (ug/L)	No. of Tanks	Mean (%)	Median (%)	Min (%)	Max (%)	Standard Deviation	Standard Error
			ATZ	0.01	8	14.095	10.238	0.000	28.571	11.016	3.895
			ATZ	0.10	8	9.452	7.738	0.000	22.222	6.498	2.297
			ATZ	1.00	8	11.536	10.101	0.000	20.000	6.652	2.352
			ATZ	25.00	8	17.657	22.650	0.000	27.273	9.479	3.351
			ATZ	100.00	8	10.545	8.013	0.000	33.333	12.393	4.382
	Decreased ovarian cavity size (Grade>1)	F	E2	0.20	8	1.736	0.000	0.000	8.333	3.299	1.166
			Ctl	0.00	16	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	0.01	8	3.115	0.000	0.000	11.111	4.493	1.588
			ATZ	0.10	8	3.212	0.000	0.000	11.111	4.620	1.634
			ATZ	1.00	8	1.042	0.000	0.000	8.333	2.946	1.042
			ATZ	25.00	8	0.962	0.000	0.000	7.692	2.720	0.962
			ATZ	100.00	8	0.962	0.000	0.000	7.692	2.720	0.962
9	Increased ovarian cavity size (Grade>0)	F	E2	0.20	8	17.305	16.228	0.000	37.500	13.568	4.797
			Ctl	0.00	16	1.016	0.000	0.000	10.000	2.858	0.715
			ATZ	0.01	8	1.743	0.000	0.000	7.692	3.250	1.149
			ATZ	0.10	8	1.563	0.000	0.000	12.500	4.419	1.563
			ATZ	1.00	8	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	25.00	8	2.747	0.000	0.000	7.692	3.795	1.342

Table 2D.4. IGB EPL - Basic Summary Statistics for all Response Variables

No.	Response Variable (Grade>0 represents % in tank having any size effect; Grade>1 represents % in tank having effect with a severity level grade >1)	Sex	Chemical	Dose (ug/L)	No. of Tanks	Mean (%)	Median (%)	Min (%)	Max (%)	Standard Deviation	Standard Error
			ATZ	100.00	8	3.184	0.000	0.000	11.111	4.567	1.615
	Increased ovarian cavity size (Grade>1)	F	E2	0.20	8	2.220	0.000	0.000	12.500	4.544	1.606
			Ctl	0.00	16	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	0.01	8	0.962	0.000	0.000	7.692	2.720	0.962
			ATZ	0.10	8	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	1.00	8	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	25.00	8	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	100.00	8	0.000	0.000	0.000	0.000	0.000	0.000
10	Ovarian interstitial fibrosis (Grade>0)	F	E2	0.20	8	5.309	6.250	0.000	11.111	4.726	1.671
			Ctl	0.00	16	5.822	0.000	0.000	30.000	9.076	2.269
			ATZ	0.01	8	7.746	6.905	0.000	14.286	5.862	2.072
			ATZ	0.10	8	4.678	3.333	0.000	12.500	5.347	1.890
			ATZ	1.00	8	4.869	0.000	0.000	18.750	7.251	2.564
			ATZ	25.00	8	5.166	7.418	0.000	11.111	4.447	1.572
			ATZ	100.00	8	0.735	0.000	0.000	5.882	2.080	0.735
	Ovarian interstitial fibrosis (Grade>1)	F	E2	0.20	8	2.134	0.000	0.000	6.250	2.957	1.045
			Ctl	0.00	16	0.347	0.000	0.000	5.556	1.389	0.347

Table 2D.4. IGB EPL - Basic Summary Statistics for all Response Variables

No.	Response Variable (Grade>0 represents % in tank having any size effect; Grade>1 represents % in tank having effect with a severity level grade >1)	Sex	Chemical	Dose (ug/L)	No. of Tanks	Mean (%)	Median (%)	Min (%)	Max (%)	Standard Deviation	Standard Error
			ATZ	0.01	8	1.786	0.000	0.000	14.286	5.051	1.786
			ATZ	0.10	8	1.389	0.000	0.000	11.111	3.928	1.389
			ATZ	1.00	8	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	25.00	8	0.893	0.000	0.000	7.143	2.525	0.893
			ATZ	100.00	8	0.000	0.000	0.000	0.000	0.000	0.000
11	Mononuclear cell infiltrates in ovary (Grade>0)	F	E2	0.20	8	32.143	36.932	15.385	45.455	10.370	3.666
			Ctl	0.00	16	31.147	28.571	11.111	64.286	14.645	3.661
			ATZ	0.01	8	29.515	28.968	13.333	43.750	13.106	4.634
			ATZ	0.10	8	39.365	35.417	30.000	57.143	10.101	3.571
			ATZ	1.00	8	32.156	34.848	16.667	44.444	10.005	3.537
			ATZ	25.00	8	22.062	23.077	0.000	38.462	11.930	4.218
			ATZ	100.00	8	36.500	34.314	20.000	55.556	12.824	4.534
	Mononuclear cell infiltrates in ovary (Grade>1)	F	E2	0.20	8	4.080	2.778	0.000	12.500	4.818	1.703
			Ctl	0.00	16	2.974	0.000	0.000	11.111	4.079	1.020
			ATZ	0.01	8	2.222	0.000	0.000	11.111	4.283	1.514
			ATZ	0.10	8	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	1.00	8	1.042	0.000	0.000	8.333	2.946	1.042
			ATZ	25.00	8	0.962	0.000	0.000	7.692	2.720	0.962

Table 2D.4. IGB EPL - Basic Summary Statistics for all Response Variables

No.	Response Variable (Grade>0 represents % in tank having any size effect; Grade>1 represents % in tank having effect with a severity level grade >1)	Sex	Chemical	Dose (ug/L)	No. of Tanks	Mean (%)	Median (%)	Min (%)	Max (%)	Standard Deviation	Standard Error
			ATZ	100.00	8	4.145	0.000	0.000	15.385	6.177	2.184
12	One or more immature ovaries	F	E2	0.20	8	2.344	0.000	0.000	12.500	4.650	1.644
			Ctl	0.00	16	4.340	0.000	0.000	21.429	6.664	1.666
			ATZ	0.01	8	2.560	0.000	0.000	7.143	3.536	1.250
			ATZ	0.10	8	3.289	3.125	0.000	7.143	3.527	1.247
			ATZ	1.00	8	1.042	0.000	0.000	8.333	2.946	1.042
			ATZ	25.00	8	2.885	0.000	0.000	7.692	3.981	1.408
			ATZ	100.00	8	1.042	0.000	0.000	8.333	2.946	1.042
	One or more unable to stage ovaries	F	E2	0.20	8	12.614	9.375	0.000	33.333	13.354	4.721
			Ctl	0.00	16	14.490	14.286	0.000	33.333	8.326	2.082
			ATZ	0.01	8	16.372	16.518	0.000	33.333	10.171	3.596
			ATZ	0.10	8	8.867	7.500	0.000	28.571	8.975	3.173
			ATZ	1.00	8	10.458	10.795	0.000	22.222	7.889	2.789
			ATZ	25.00	8	10.080	8.392	0.000	23.077	6.800	2.404
			ATZ	100.00	8	11.570	9.722	0.000	30.000	8.948	3.164
13	Fused kidneys (Grade>0)	F	E2	0.20	8	19.446	17.424	12.500	36.364	7.281	2.574
			Ctl	0.00	16	13.827	11.111	0.000	35.714	11.841	2.960
			ATZ	0.01	8	14.921	20.000	0.000	35.714	13.350	4.720

Table 2D.4. IGB EPL - Basic Summary Statistics for all Response Variables

No.	Response Variable (Grade>0 represents % in tank having any size effect; Grade>1 represents % in tank having effect with a severity level grade >1)	Sex	Chemical	Dose (ug/L)	No. of Tanks	Mean (%)	Median (%)	Min (%)	Max (%)	Standard Deviation	Standard Error
			ATZ	0.10	8	17.991	14.881	0.000	50.000	16.272	5.753
			ATZ	1.00	8	16.256	13.889	0.000	41.667	14.869	5.257
			ATZ	25.00	8	11.691	12.698	0.000	30.769	9.862	3.487
			ATZ	100.00	8	18.623	11.806	0.000	40.000	15.989	5.653
		M	E2	0.20	8	18.264	5.556	0.000	60.000	24.436	8.640
			Ctl	0.00	16	10.175	9.545	0.000	30.000	10.306	2.577
			ATZ	0.01	8	11.643	10.101	0.000	25.000	7.575	2.678
			ATZ	0.10	8	12.739	12.917	0.000	20.000	5.978	2.113
			ATZ	1.00	8	13.691	12.698	6.667	23.077	6.293	2.225
			ATZ	25.00	8	13.310	9.545	0.000	30.000	10.204	3.607
			ATZ	100.00	8	21.489	20.913	6.667	41.667	10.969	3.878
	Fused kidneys (Grade>1)	F	E2	0.20	8	0.694	0.000	0.000	5.556	1.964	0.694
			Ctl	0.00	16	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	0.01	8	0.833	0.000	0.000	6.667	2.357	0.833
			ATZ	0.10	8	0.781	0.000	0.000	6.250	2.210	0.781
			ATZ	1.00	8	1.136	0.000	0.000	9.091	3.214	1.136
			ATZ	25.00	8	0.893	0.000	0.000	7.143	2.525	0.893
			ATZ	100.00	8	0.000	0.000	0.000	0.000	0.000	0.000
		M	E2	0.20	8	0.000	0.000	0.000	0.000	0.000	0.000

Table 2D.4. IGB EPL - Basic Summary Statistics for all Response Variables

No.	Response Variable (Grade>0 represents % in tank having any size effect; Grade>1 represents % in tank having effect with a severity level grade >1)	Sex	Chemical	Dose (ug/L)	No. of Tanks	Mean (%)	Median (%)	Min (%)	Max (%)	Standard Deviation	Standard Error
			Ctl	0.00	16	0.568	0.000	0.000	9.091	2.273	0.568
			ATZ	0.01	8	1.918	0.000	0.000	9.091	3.631	1.284
			ATZ	0.10	8	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	1.00	8	1.923	0.000	0.000	15.385	5.439	1.923
			ATZ	25.00	8	1.250	0.000	0.000	10.000	3.536	1.250
			ATZ	100.00	8	0.962	0.000	0.000	7.692	2.720	0.962
14	Mineralization (Grade>0)	F	E2	0.20	8	12.058	8.013	0.000	50.000	16.161	5.714
			Ctl	0.00	16	19.798	17.692	0.000	40.000	11.794	2.948
			ATZ	0.01	8	12.370	13.810	0.000	22.222	7.229	2.556
			ATZ	0.10	8	22.272	23.611	13.333	33.333	6.475	2.289
			ATZ	1.00	8	24.187	26.136	8.333	36.364	10.540	3.726
			ATZ	25.00	8	16.345	15.385	0.000	44.444	13.505	4.775
			ATZ	100.00	8	28.913	24.038	6.667	66.667	18.524	6.549
		M	E2	0.20	8	10.139	0.000	0.000	50.000	17.732	6.269
			Ctl	0.00	16	16.110	15.476	0.000	30.769	10.621	2.655
			ATZ	0.01	8	12.432	10.101	0.000	27.273	8.716	3.082
			ATZ	0.10	8	24.282	24.747	0.000	50.000	17.692	6.255
			ATZ	1.00	8	31.852	35.897	7.692	54.545	17.330	6.127
			ATZ	25.00	8	21.256	20.629	10.000	30.000	6.675	2.360

Table 2D.4. IGB EPL - Basic Summary Statistics for all Response Variables

No.	Response Variable (Grade>0 represents % in tank having any size effect; Grade>1 represents % in tank having effect with a severity level grade >1)	Sex	Chemical	Dose (ug/L)	No. of Tanks	Mean (%)	Median (%)	Min (%)	Max (%)	Standard Deviation	Standard Error
			ATZ	100.00	8	23.961	22.500	0.000	42.857	13.577	4.800
	Mineralization (Grade>1)	F	E2	0.20	8	0.000	0.000	0.000	0.000	0.000	0.000
			Ctl	0.00	16	0.481	0.000	0.000	7.692	1.923	0.481
			ATZ	0.01	8	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	0.10	8	1.823	0.000	0.000	8.333	3.421	1.210
			ATZ	1.00	8	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	25.00	8	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	100.00	8	0.000	0.000	0.000	0.000	0.000	0.000
		M	E2	0.20	8	0.000	0.000	0.000	0.000	0.000	0.000
			Ctl	0.00	16	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	0.01	8	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	0.10	8	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	1.00	8	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	25.00	8	1.136	0.000	0.000	9.091	3.214	1.136
			ATZ	100.00	8	0.000	0.000	0.000	0.000	0.000	0.000
15	Other renal diagnoses (Grade>0)	F	E2	0.20	8	0.658	0.000	0.000	5.263	1.861	0.658
			Ctl	0.00	16	0.446	0.000	0.000	7.143	1.786	0.446
			ATZ	0.01	8	3.460	0.000	0.000	14.286	5.324	1.882

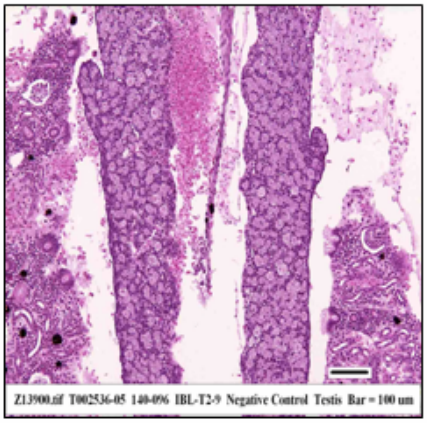
Table 2D.4. IGB EPL - Basic Summary Statistics for all Response Variables

No.	Response Variable (Grade>0 represents % in tank having any size effect; Grade>1 represents % in tank having effect with a severity level grade >1)	Sex	Chemical	Dose (ug/L)	No. of Tanks	Mean (%)	Median (%)	Min (%)	Max (%)	Standard Deviation	Standard Error
			ATZ	0.10	8	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	1.00	8	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	25.00	8	1.923	0.000	0.000	15.385	5.439	1.923
			ATZ	100.00	8	1.563	0.000	0.000	12.500	4.419	1.563
		M	E2	0.20	8	0.000	0.000	0.000	0.000	0.000	0.000
			Ctl	0.00	16	0.833	0.000	0.000	6.667	2.277	0.569
			ATZ	0.01	8	1.563	0.000	0.000	12.500	4.419	1.563
			ATZ	0.10	8	1.389	0.000	0.000	11.111	3.928	1.389
			ATZ	1.00	8	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	25.00	8	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	100.00	8	0.000	0.000	0.000	0.000	0.000	0.000
	Other renal diagnoses (Grade>1)	F	E2	0.20	8	0.658	0.000	0.000	5.263	1.861	0.658
			Ctl	0.00	16	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	0.01	8	0.781	0.000	0.000	6.250	2.210	0.781
			ATZ	0.10	8	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	1.00	8	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	25.00	8	0.962	0.000	0.000	7.692	2.720	0.962
			ATZ	100.00	8	0.000	0.000	0.000	0.000	0.000	0.000

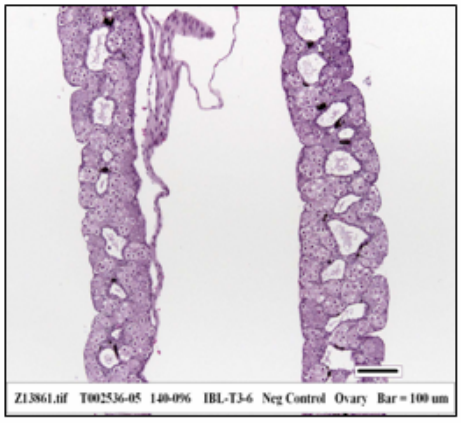
Table 2D.4. IGB EPL - Basic Summary Statistics for all Response Variables

No.	Response Variable (Grade>0 represents % in tank having any size effect; Grade>1 represents % in tank having effect with a severity level grade >1)	Sex	Chemical	Dose (ug/L)	No. of Tanks	Mean (%)	Median (%)	Min (%)	Max (%)	Standard Deviation	Standard Error
		M	E2	0.20	8	0.000	0.000	0.000	0.000	0.000	0.000
			Ctl	0.00	16	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	0.01	8	1.563	0.000	0.000	12.500	4.419	1.563
			ATZ	0.10	8	1.389	0.000	0.000	11.111	3.928	1.389
			ATZ	1.00	8	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	25.00	8	0.000	0.000	0.000	0.000	0.000	0.000
			ATZ	100.00	8	0.000	0.000	0.000	0.000	0.000	0.000

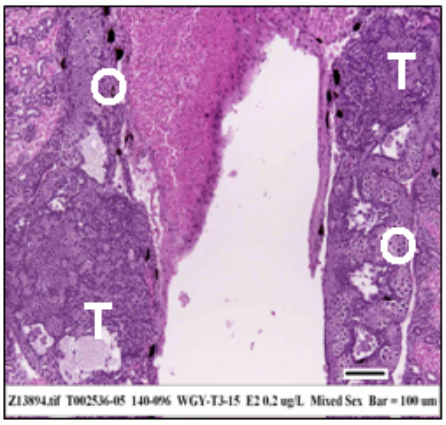
APPENDIX 3. DIGITAL IMAGES OF SELECTED HISTOLOGICAL ENDPOINTS



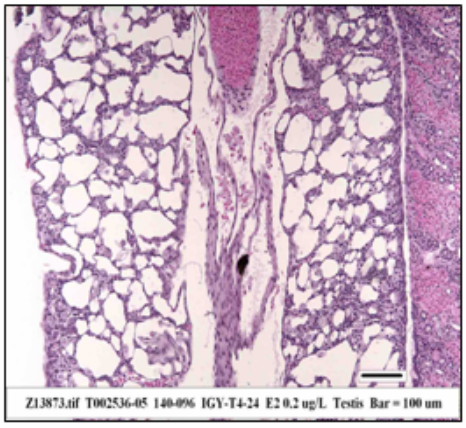
Testis, Unremarkable/Normal



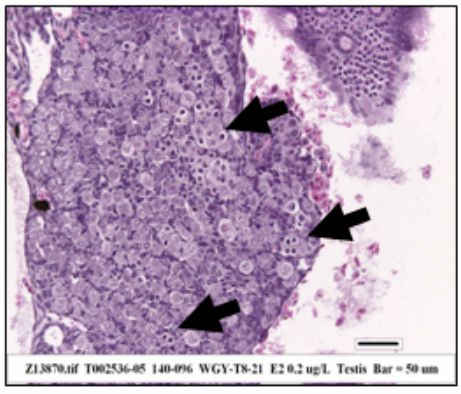
Ovary, Unremarkable/Normal



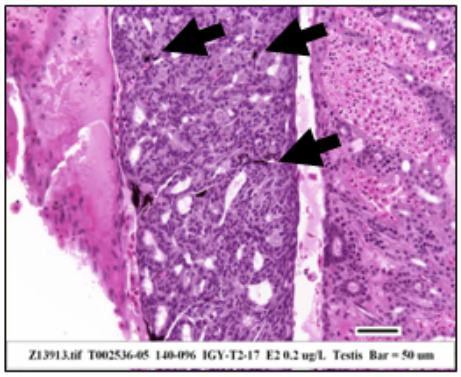
Mixed Sex: The presence of opposite sex tissue in the testis or ovary.



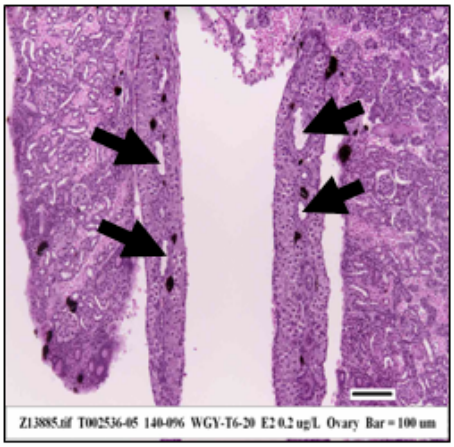
Dilated tubules: The presence of multiple, irregularly enlarged tubules within a testis.



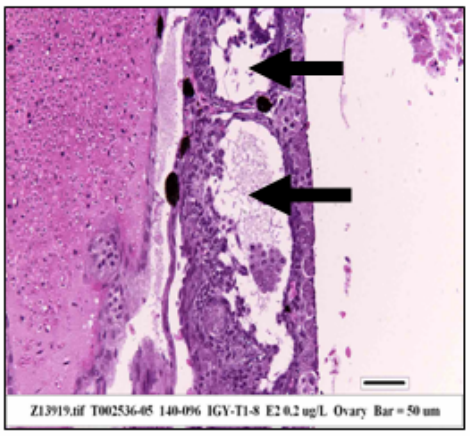
Dividing Gonocytes: The presence in the testis of one or more clusters of germ cells that have pale or clear cytoplasm and a dense, hyperchromatic nuclei with slightly irregular margins. These resemble mitotically-active primordial germ cells that are common in post-metamorphic ovaries.



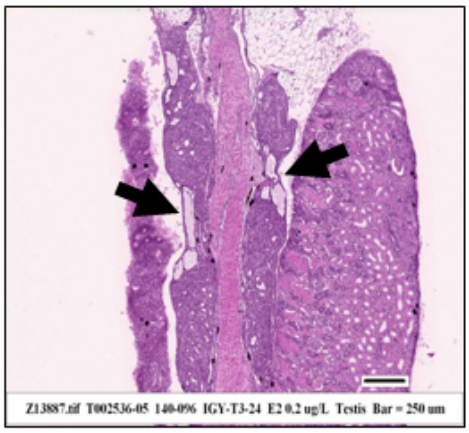
Internal Melanophores: The presence of one or more small oval or polygonal cells with solid dark brown cytoplasmic staining, or large stellate cells with long cytoplasmic processes that contain medium brown granular pigment. By convention, internal melanophores are those that are located within the testis parenchyma at least one spermatogonial cell's width from the surface.



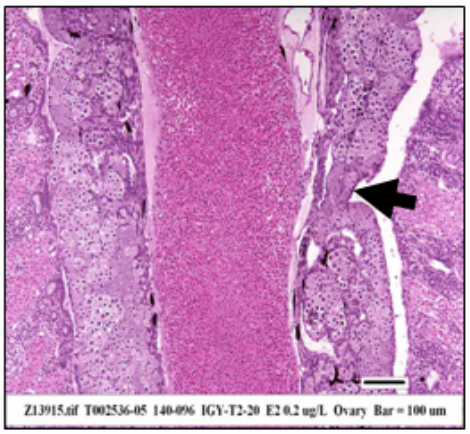
Ovarian Cavity, Decreased: A substantial reduction in the size of the central ovarian cavity, among all examined sections, as compared to ovarian cavities in the majority of control females.



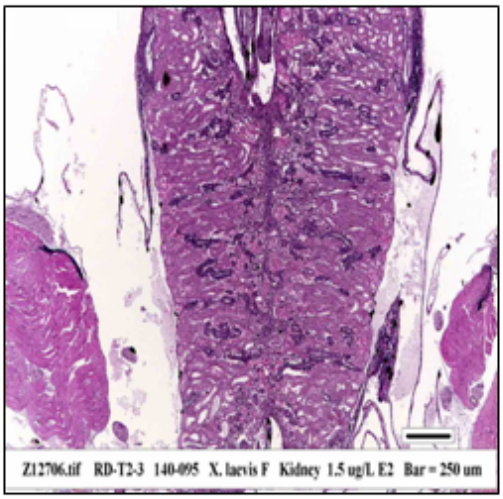
Ovarian Cavity, Increased: A substantial enlargement in the size of the ovarian cavity, among all examined sections, as compared to ovarian cavities in the majority of control females. In addition to being enlarged, an affected ovarian cavity may have a "ragged" irregular contour.



Segmental Hypoplasia (testis): A substantial (e.g., > 1/2 diameter), abnormal, focal or multifocal decrease in the width or thickness of the testis. Because illusory narrowing may be seen when viewing the three-dimensional testis in two-dimensional histologic sections, the diagnosis of segmental hypoplasia is often prompted and/or confirmed by necropsy findings and gross photographs.



Segmental Hypoplasia, Ovary: A substantial (e.g., > 1/2 diameter), abnormal, focal or multifocal decrease in the width or thickness of the ovary. Because illusory narrowing may be seen when viewing the three-dimensional ovary in two-dimensional histologic sections, the diagnosis of segmental hypoplasia is often prompted and/or confirmed by necropsy findings and gross photographs.



Fused Kidneys: A conjoining of the right and left kidneys. For purposes of this study, right and left kidneys are considered conjoined if they appear to share any tubular elements. Right and left kidneys that are adjoined by only membranous attachments are excluded from this diagnosis.