Gonadal Development of Larval Male *Xenopus laevis* Exposed to Atrazine in Outdoor Microcosms

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The potential effects of atrazine on gonadal development in metamorphs and subadults of the African clawed frog (*Xenopus laevis*) were studied under conditions of natural photoperiod and temperatures in outdoor microcosms from August 2002 to June 2003 in South Africa. Triplicate 1100 L microcosms for each nominal concentration of 0.0, 1, 10, and 25 μg of atrazine/L were used. Measured atrazine concentrations varied <25% throughout the study, and no atrazine was detected in the control microcosms. Tadpoles developed well at all concentrations. On the basis of histological examination of testes of recently metamorphosed stage 66 frogs, 57% of the individuals in the reference group exhibited testicular oocytes as compared with 57, 59, and 39% of the 1, 10, and 25 μg/L atrazine groups, respectively. The average prevalence of testicular oocytes for all of the treatments including the controls was 54% in a single testis, while, in 35% of individuals, testicular oocytes were observed in both testes. The number of testicular oocytes per individual ranged from 0 to 58 with means of 9.5, 9.8, 8.5, and 11.1 for the 0.0, 1, 10, and 25 μg of atrazine/L groups, respectively. Ten months after metamorphosis, another subset of juveniles was examined, and the maximum number of testicular oocytes observed was five in one animal. The presence of testicular oocytes was not related to exposure to atrazine and may be a natural phenomenon during ontogeny.

Introduction

Xenopus laevis has been used for decades as a model for studying the hormonal control of gonadal differentiation on and the impact of contaminants on this process (*1*). During normal development, the gonads can be first recognizable at the Nieuwkoop−Faber stage 46 (*2*, *3*). Primordial germ cells are visible in the genital ridge as early as NF stage 40 and, by NF stage 49, have moved into the developing gonad. As the gonad develops, the interior medulla regresses in female frogs, forming a conspicuous ovarian cavity, whereas in males the cortex regresses after primordial germs cells have migrated into the medulla. By completion of metamorphosis (stage 66), the gonads are clearly differentiated into ovaries and testes that can be distinguished at the gross morphological level.

Numerous studies have shown that treatment of *X. laevis* larvae with exogenous estradiol (*4*−*8*) or other contaminants (*9*−*12*) can affect normal gonadal differentiation, in some cases leading to ovary formation in males or the development of intersexual gonads that contain both testicular and ovarian tissue. Several recent studies have suggested that exposure to the triazine herbicide, atrazine, during larval development may affect gonadal differentiation in frogs, although the threshold concentration for this response is unclear (*13*−*15*). In a laboratory study where *X. laevis* larvae were exposed from hatching to metamorphosis to atrazine concentrations from 0.1 to 200 μg/L, no effects on larval growth, developmental rate, mortality, time to metamorphosis, or size at metamorphosis in females or males were observed (*16*). However, it was reported that atrazine exposure reduced the size of the male laryngeal dilator muscle and resulted in gonadal deformities (*16*), including the presence of testicular oocytes (*13*). The former effect was suggested to occur through an androgen-dependent mechanism at concentrations ≥1 μg/L of atrazine (*16*), while the latter effect was suggested to occur through an estrogenic mechanism of action. Exposure to concentrations as small as 0.1 μg/L also has been reported to induce gonadal anomalies in male *X. laevis* (*16*) and in male *Rana pipiens* in laboratory and field studies (*13*). Other studies of the potential effects of atrazine on *X. laevis* have failed to demonstrate any effects on laryngeal dilator muscle (*14*, *15*) or the prevalence of testicular oocytes (*15*); however, in one of these studies, animals exposed to 25 μg/L of atrazine exhibited a statistically significant greater frequency of discontinuous and intersexual gonads as compared to animals raised at concentrations <25 μg/L of atrazine (*14*). Discontinuous gonads and other testicular anomalies have been reported in other laboratory studies on *X. laevis* and *Rana clamitans* (*15*, *17*) and also in the field in *X. laevis* (*18*, *19*), but no responses to exposure concentrations of atrazine were observed. To date, no study has reported the presence of testicular oocytes in *X. laevis* under natural conditions.

While effects were observed in frogs exposed to atrazine under field conditions and statistically significant changes in biomarkers such as steroid hormone titers were observed by Hecker et al. (*20*), these responses were not consistent with those based on the theory of induction of aromatase suggested by Hayes et al. (*16*). Furthermore, the effects observed by Hecker et al. (*20*) could not be attributed solely to atrazine and may have been confounded by exposures to

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other stressors or by changing concentrations resulting from rainfall and runoff events.

Historical observations have suggested that the occurrence of intersex and other gonadal anomalies in frogs from various habitats are commonly found at low frequencies (1−11%) (21) but occasionally at frequencies >35% (22). Some of these observations preceded the patenting and initial U.S. registration of atrazine in 1957 or other synthetic pesticides. A retrospective study using museum specimens of the cricket frog (*Acris clepitans*) conducted by Reeder et al. (21) found gonadal abnormalities in individuals collected before the use of either atrazine or other synthetic organochlorines, but the authors suggested that there may have been an increase in the prevalence that was related to concentrations of organochlorine compounds in the environment.

Although many community-based controlled toxicology studies in semifield conditions have been performed to identify direct and indirect effects of atrazine (23), only a few have involved amphibians (24). Semifield studies, such as in outdoor microcosms, offer the advantages of observing large numbers of animals under more natural conditions of temperature and photoperiod than can be achieved under indoor laboratory conditions, while exposures to the test substance and potentially confounding stressors can be more closely controlled and monitored than in the field. Furthermore, due to ubiquitous low-level concentrations of atrazine in the field, the mesocosm study allowed the study of the development of *X. laevis* under natural conditions in the absence of measurable concentrations of atrazine. For these reasons, a semifield microcosm study was conducted to determine the effect, if any, of atrazine at different concentrations on the number of days to metamorphosis and gonadal development of *X. laevis* up to and beyond developmental stage 66 (25).

Materials and Methods

Experimental Design. Twelve outdoor in-ground microcosms, 2.25 m long, 1.2 m wide, and 1.0 m deep and lined with a polyethylene membrane were constructed at the experimental facility of the North-West University, Potchefstroom Campus, South Africa. A 20 cm high wall around each microcosm prevented surface water runoff from entering the microcosms. The bottoms of the microcosms were covered with a 3 cm layer of sandy soil, and they were filled with 1100 L of dechlorinated city water. The water depth was recorded and maintained throughout the study by the addition of tap water as required. Microcosms were covered with hail netting to exclude predators such as dragonflies and birds. Macrophytes (*Ceratophylum*) from field sites were introduced and allowed to colonize the microcosms for 5 months. In August 2002, 12 microcosms were randomly allocated in three replicates of four experimental treatments (including the control). One set of three microcosms received no atrazine and served as references. Three microcosms per concentration were initially treated with atrazine dissolved in methanol to achieve initial nominal concentrations of 1, 10, and 25 μg/L. The concentration of methanol was 250 μL/L (v/v), and the same amount of methanol was added to all treated and reference microcosms.

Animals. Seven pairs of adult *X. laevis* in reproductive condition were captured from a field site where no atrazine was applied in the watershed. This field site is a large earth walled pond with muddy water sustaining a large population of *X. laevis.* Frogs were captured using baited bucket traps and kept in dechlorinated tap water at room temperature and exposed to daylight for 1 week. Frogs were not fed prior to spawning. Spawning was induced using commercially available chorionic gonadotrophin (GC; Pregnyl, Donmed Pharmaceuticals (Pty) Ltd, Bedfordview, RSA). Four male frogs were injected subcutaneously in the dorsal lymph sack with 250 IU of GC for three consecutive days, while four females were injected on days two and three only with 50 and 500 IU of GC, respectively. After the final injection, frog pairs were placed in breeding tanks lined with a polyethylene mesh (6 mm) covering the bottom to protect the eggs and placed in a dark room. The following morning, the adult frogs were removed, and the water was aerated. After hatching, tadpoles from all breeding tanks were transferred to one tank. One hundred actively swimming 4 day old tadpoles were siphoned from the tank into glass jars using a glass tube connected to a silicone tube. This process was repeated until each of the 12 jars contained 600 tadpoles. Tadpoles were released into the microcosms. Two weeks later, an additional 200 4 day old tadpoles produced by the previous method were released into each of the microcosms to achieve an initial population of 800 tadpoles. All animal-use protocols were approved by the Animal Care and Use Committee of the North-West University.

Data Collection. Water quality parameters including dissolved oxygen, conductivity, pH, air, and water temperatures were recorded weekly using a YSI 556 multi-probe system data-logger (YSI Environmental, Baton Rouge, LA). Bi-weekly, 1 L water samples were collected from each microcosm to measure the atrazine concentrations. A single scan was conducted at the initiation of the study to determine elemental concentrations in water and sediment samples.
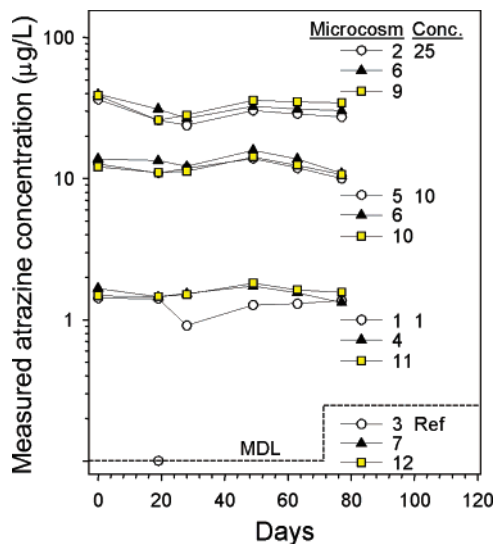
Atrazine concentrations in the microcosms were measured by extraction from water with C18-SPE columns and subsequent quantification with an Agilent model 6890 series II^{plus} gas chromatograph interfaced to a 5973 mass selective detector (GC/MSD) operated in the selected ion monitoring (SIM) mode. The method detection limit (MDL) was 0.1 μg/L for atrazine (19).

Feeding. Algae and zooplankton flourished in the microcosms, but supplementary food (Complete Rabbit Pellets: Epol, Pretoria, RSA) was initially given once a week and, later in the study, twice a week. The food supplement per microcosm consisted of 20 g of pulverized rabbit pellets, homogenized in 500 mL of tap water. The rabbit pellets contained 160 g/kg of protein, 25 g/kg of fat, 170 g/kg of fiber, 18 g/kg of calcium, and 7 g/kg of phosphorus.

Development of Metamorphs. Extensive mortality occurred in microcosms one and eight (1 and 10 μg/L of atrazine, respectively) early in the study. The cause of the mortality could not be identified, but since it did not occur in other replicate microcosms of these treatments, it was not attributable to atrazine exposure. These two microcosms were subsequently excluded from the study. Metamorphs reaching developmental stage 66 were removed from the microcosms every second day from November 2002 until mid-January 2003. A total of 150 metamorphs reaching developmental stage 66 were removed from the microcosms for each treatment concentration. It was not possible to obtain 50 specimens from each of the three microcosms per treatment group, but no more than 75 metamorphs were collected from a single microcosm replicate.

Collected specimens were anaesthetized by immersion in a 1:1000 dilution of 3-amino-benzoic-acid-ethyl-ester (MS-222). The date of completion of metamorphosis was recorded and the body mass was determined. Snout−vent length was measured to the nearest 0.1 mm. A small cut was made on the abdomen to allow penetration of the fixative, and an identification tag was attached. Specimens were fixed in Bouin's fixative for 48 h, rinsed in water, and transferred to 70% EtOH. All the frogs were then dissected to expose the gonads for gross morphological inspection. The gender of each frog was determined, and the gonads were digitally photographed.

Histology. Frogs were collected for histological examination from each treatment group at two distinct time periods,

FIGURE 1. Atrazine concentrations as measured in the microcosms. Each point represents an analysis of a single microcosm. Changes in concentration resulted from evaporation and rainfall events during the study.

at developmental stage 66 and at 10 months of age. A total of 214 randomly selected stage 66 metamorphs from the control and the three atrazine concentrations were histologically examined. Of these, 45 were from the reference ponds (23 males and 22 females), 54 from 1 μg/L (30 males and 24 females), 51 from 10 μg/L (17 males and 34 females), and 58 from 25 μg/L (31 males and 27 females) ponds. The preserved gonads were dehydrated in graded alcohols and embedded in paraffin. Serial sections (6 μm) of the entire gonadal area were cut and stained with Meyer hematoxylin and eosin. Each section was examined for anomalies.

Results

Atrazine Concentrations in the Microcosms. Atrazine concentrations in the microcosms remained consistent throughout the first 80 days of the study, and it was not necessary to add more atrazine at any stage (Figure 1). Thereafter, the microcosms were drained, and the water and atrazine were replaced. Atrazine concentrations were monitored, and frogs were grown out to maturity as part of a further study. Concentrations fluctuated in response to evaporation of water from the microcosms, rainfall, and addition of water to maintain a consistent depth. Measured concentrations of atrazine in the three treatments were somewhat greater than the nominal values with time weighted means of 1.43, 12.1, and, 30.8 μg of atrazine/L, respectively. Atrazine was not detectable in the control microcosms except on one occasion, when a positive signal was observed in one of the reference microcosms. The area of the peak was equivalent to the method detection limit of 0.1 μg/L and thus was not a quantifiable concentration. However, to be certain that there was no atrazine-caused effect in the references, no specimens from this microcosm were used in the study.

Elements and Major Ions in Water and Sediment. The temperature, pH, electrical conductivity (EC), and dissolved oxygen (DO) are shown in Figure 2. Measurement of the elements and major ions in water and sediment revealed high concentrations of calcium, magnesium, sodium, and sulfur (see Table 1 in Supporting Information). All parameters were within water-quality guidelines (26, 27).

Development of *X. laevis* Tadpoles and Gross Morphology. Although the microcosms were cooler than most natural water bodies in the Potchefstroom region, tadpoles schooled
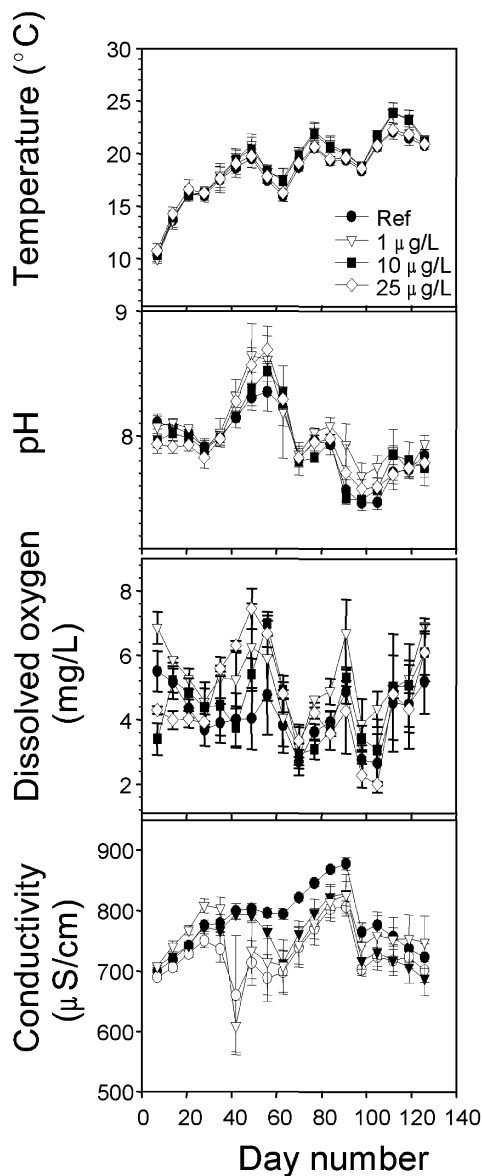
FIGURE 2. Mean temperature, pH, dissolved oxygen, and conductivity of the microcosms. Bars represent standard error of the mean.

normally and appeared to not be under stress. The first metamorphs reached developmental stage 66 in the 10th week of the study. From then on, the number of frogs reaching stage 66 increased gradually. Collection of stage 66 metamorphs was terminated in week 19 when the target numbers were met.

A number of terms has been used to describe anomalies of the gonads in frogs (28). At the gross morphological level, the ambiguous sex, where there is ovarian and testicular tissue in the same animal that is segregated laterally or rostral/caudal, has been referred to as hermaphrodite (16) and as intersex (14). Ambiguous sex where ovarian and testicular tissue is mixed in the same gonad has been referred to as hermaphrodite (16), intersex, (14), and sex reversal (13). Abnormal segmentation in gonad and/or segments of gonad separated by undifferentiated tissue has been referred to as multiple gonads (16) and discontinuous gonad (14). Retarded gonadal development and underdeveloped testes have been referred to as testicular dysgenesis (13). At the histological level, the condition of low to absent germ cells, testicular tubules that are poorly developed, has been referred to as testicular dysgenesis (13). The presence of oocytes in the testes (small numbers of stage I previtellogenic <300 μm

TABLE 1. Number of Stage 66 Metamorphs Examined with the Number and Percentage of Macroscopic Gonadal Anomalies Observed Visually
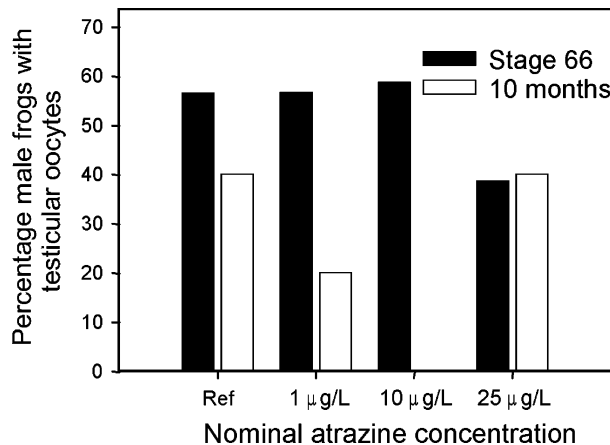
nominal (time weighted mean, range of measured) concentrations in μg/L	number of				percent gross gonadal anomalies	
	frogs collected	males	females	males with anomalies	total frogs	males
reference	150	68	82	6	4.0	8.8
1 (1.4, 0.91−1.82)	150	72	78	2	1.3	2.8
10 (12.1, 10−15.9)	150	59	91	1	0.7	1.7
25 (30.8, 23.8−39.7)	150	70	80	5	3.3	7.1
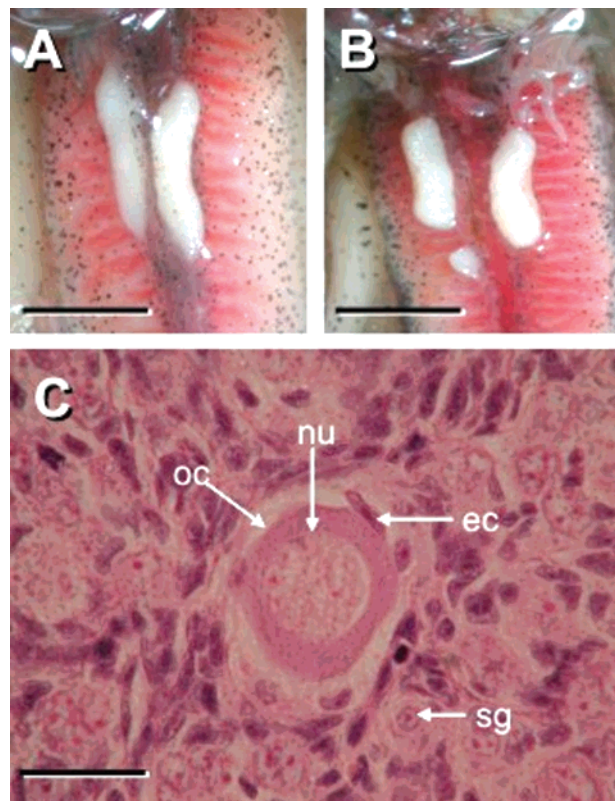
FIGURE 3. (A) Normal testis. (B) Macroscopic testicular anomaly. (C) Micrograph of a testicular oocyte. Abbreviations: ec, epithelial cell; oc, oocyte; nu, nucleus; and sg, spermatogonium. Scale bars: (A and B) 3 mm and (C) 30 μm.

FIGURE 4. Prevalence of testicular oocytes in the different atrazine exposure concentrations in stage 66 and 10 month old male frogs. The $X2$ test showed no significant difference between treatments.

diameter, intact nucleus with nucleoli, surrounded by squamous epithelium (*29*)) is referred to as testicular oocytes in this paper but also as testicular oogenesis by others (*13*). Large numbers of oocytes in the testis, some of which may be vitellogenic, have been referred to as sex reversal (*13*). For the purposes of discussion in this paper, all of these conditions observed in male frogs are referred to by the general term gonadal anomalies. To avoid confusion, photographs are presented to demonstrate each specific condition (Figure 3).

Of the 150 stage 66 metamorphs visually examined in each treatment concentration, gross macroscopic gonadal anomalies (Figure 3) were few, no concentration response was observed (Table 1), and no intersex gonads were observed. All of the gonadal anomalies were observed only in males and consisted only of abnormal segmentation (Table 1 and Figure 3). The cause of these macroscopic anomalies is not known. They may be the result of delayed maturation of the testis, but the fact that that they were also seen in similar low frequency (2%) in *X. laevis* adults from the field (*18*) suggests that this is not the case.

Histological Examination. The only type of gonadal anomaly observed during histological examination was the presence of testicular oocytes (Figure 3).

These testicular oocytes were identified as stage 1 oocytes since the nucleus of the chromosomes had assumed the lamp−brush configuration. The extra nucleoli are also sequestered at the outer border of the nucleus, a follicular cell layer was present, and the cytoplasm had a basophilic character. The testicular oocytes were small and ranged in diameter from 29 to 40 μm.

The prevalence of individuals with testicular oocytes was 57% in the reference frogs, as compared to 57% in frogs exposed to 1 μg/L, 59% in frogs exposed to 10 μg/L of atrazine, and 39% in frogs exposed to 25 μg/L (Figure 4). There was no statistically significant difference between the prevalence of testicular oocytes among treatments ($X^2 = 2.94$, 3 d.f., $p = 0.40$). Also, there was no correlation between gross morphological anomalies and the presence of testicular oocytes. No gonadal anomalies were observed histologically in any of the female metamorphs.

Testicular oocytes were found in one testis in some individuals and in both testes in others. The same trend was observed in the control and at all the atrazine concentrations. In frogs not exposed to atrazine, 54% of individuals had testicular oocytes in both of the testes, while in frogs exposed to 1 μg/L, 35% had testicular oocytes in both of the testes. In the frogs exposed to 10 μg/L, 60% had testicular oocytes in both testes and, in frogs exposed to 25 μg/L, 50% had testicular oocytes in both of the testes. There was no statistically significant difference between the prevalence of testicular oocytes in only one or both testes ($X^2 = 1.87$, 3 d.f., $p = 0.60$).

There was no statistically significant difference in the number of testicular oocytes in the gonads of frogs from any of the treatments (Kruskal−Wallis test; $p < 0.27$). The mean number of testicular oocytes per specimen was 8.5 in frogs
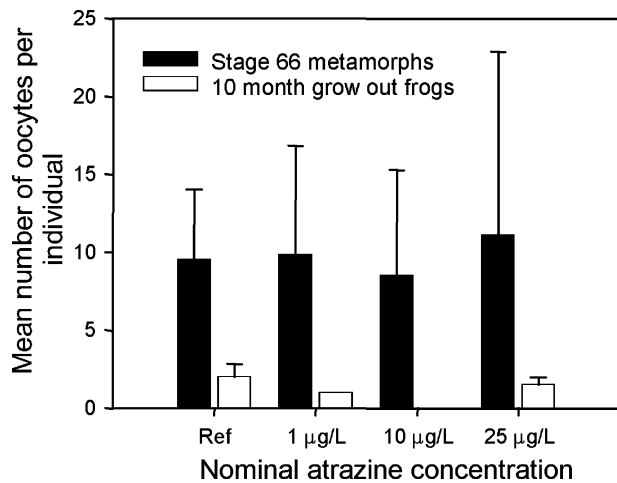
FIGURE 5. Mean number of testicular oocytes found per specimen in the reference and atrazine exposed stage 66 and 10 month old juvenile male frogs. Bars represent 95% confidence intervals.

exposed to 10 μg/L and 11.1 in frogs exposed to 25 μg/L (Figure 5). Numbers of testicular oocytes per specimen in the reference frogs and frogs exposed to 1 μg/L were 9.5 and 9.8, respectively. The majority of individuals expressing testicular oocytes had between two and 10 testicular oocytes per testis. A maximum of 58 testicular oocytes was observed in one individual.

The prevalence of testicular oocytes as well as the number of testicular oocytes per individual was less in the 10 month old individuals than the stage 66 individuals (Figures 4 and 5). The maximum number of testicular oocytes per specimen was five, as compared to 58 in stage 66 individuals.

Discussion

The slower development of tadpoles as observed in the study is not surprising since the water temperatures in the microcosms (10 °C in the first week rising to 20 °C in week 10) were generally less than those observed at equivalent times in natural ponds in the region. Under controlled temperature conditions of 20−25 °C, *X. laevis* tadpoles take approximately 58 days to complete metamorphosis (*25*). In its natural habitat, where temperatures fluctuate, time to metamorphosis for *X. laevis* ranges from 56 to 63 days (*30*). The slower development in the present study was most likely a result of low water temperature. The microcosms were covered with hail netting and partially shaded by trees and a building. Furthermore, the ponds had a uniform depth, such that there were no shallower parts where the water temperature would be greater, which is generally the case in natural ponds. The lethal temperatures for *X. laevis* embryos has been reported to be greater than 35 °C and less than 10 °C (*30*). It is highly unlikely that density of tadpoles per microcosm had a significant effect on development. The initial density was less than one tadpole per liter of water, and with natural mortality, the volume of water per tadpole would have increased. The *X. laevis* tadpoles school and have been observed in natural water bodies in far greater densities than in the microcosms (L. Du Preez, personal communication).

Although testicular oocytes were observed, no ambiguous-sex gonads with separate regions of ovarian and testicular tissue were observed during gross morphological examination. The frequency of gonadal anomalies in the form of abnormally segmented testes observed was small with proportions between 1.7 and 7.1% in male frogs exposed to atrazine in microcosms and 8.8% in the reference microcosms (Table 1 in Supporting Information). There was no relationship between these anomalies and testicular oocytes, which

were observed at a much larger frequency (Figure 4). The gonadal anomalies observed in frogs from the exposed microcosms showed no concentration responses to atrazine. Also, no gross gonadal anomalies were observed in frogs identified as females. On the basis of gross morphology, atrazine had no adverse effect on gonadal development at time-weighted mean measured concentrations ranging from 1.4 to 30.8 μg/L under semifield conditions.

The phenomenon of testicular oocytes has not been widely reported for *X. laevis* and may have important biological significance, especially with respect to gauging the degree of gonadal maturation in wild-caught animals. Few studies have actually conducted complete serial sectioning of every testis, which is what is required to find these 30 μm cells. We suspect that most researchers do not perform histological analysis unless gross gonadal anomalies are observed. Thus, if the incidence of gross abnormalities is low in the controls, they look no further. Intermittent or partial sampling of sections can lead to false negatives if the interval distance between sections is greater than the diameter of the oocytes. The testicular oocytes observed in the present study were determined to not be primordial germ cells, which have an average diameter of 17 μm in *Xenopus* (*31*). The testicular oocytes observed in the present study ranged in diameter from 28 to 40 μm and were smaller than the usual 50 μm reported for developing ovaries of *X. laevis* (*31*). The testicular oocytes were stage I, previtellogenic oocytes surrounded by follicular cell layers with basophilic inclusion (Figure 3).

Other authors have reported the presence of gonadal abnormalities and intersex in control and atrazine-exposed *X. laevis* metamorphs. Gonadal anomalies (discontinuous testes) and intersex were observed in control as well as atrazine-exposed *X. laevis* in a laboratory study (*14*). As compared to controls, the frequency of abnormalities and intersex was significantly increased only in animals exposed to 25 μg of atrazine/L. Gonadal anomalies at a frequency of ~6% were reported in control *X. laevis* metamorphs in a chronic exposure study with bisphenol A (*10*). Gonadal anomalies and testicular oocytes were also observed in control and atrazine-exposed *X. laevis* in another laboratory study (*15*). Frequency of occurrence (0−7% in stage 66 metamorphs) was comparable to our observations, and no concentration−response to atrazine exposures up to 25 μg/L was observed. In an ongoing study on the annual reproductive cycle of *X. laevis* conducted in South Africa, 7% of the males collected from natural sites were found to have testicular oocytes (Everson, unpublished data). An expansion of this study to include *X. laevis* collected from remote regions that are relatively free of anthropogenic contamination will be useful to better characterize this phenomenon.

Reports based on laboratory and field studies by Hayes et al. (*13, 16*) suggest that atrazine causes an increase in the frequency of abnormally segmented gonads and testicular oocytes in frogs at concentrations $\geq$0.1 μg/L and that there is no occurrence in unexposed controls. The results of the microcosm study reported here suggest that both abnormal segmentation and testicular oocytes occur in unexposed as well as atrazine-exposed frogs. In addition, there was no concentration-dependent response between the frequency of the presence of testicular oocytes and the total number of testicular oocytes in individual frogs. The reasons for the differences in results in our studies and those reported by Hayes et al. (*13, 16*) are not clear but may be due to how the prevalence of testicular oocytes were defined in the two studies. Exposures to atrazine in the microcosms were continuous and well-characterized during the entire period of the study. Although exposure concentrations in Hayes' studies were confirmed by analysis (*13, 16*), the values were not reported, so it is impossible to know the actual concentrations or the limits of quantification. The field study on

R. pipiens by Hayes et al. (*13*) only measured concentrations of atrazine when the frogs were captured, not at earlier times during sexual differentiation, making it impossible to characterize a concentration response, if any.

When considering the results of all these studies, there is little evidence to suggest that atrazine causes male gonadal anomalies in *X. laevis* at environmentally relevant concentrations. The decrease in the number of testicular oocytes observed as the frogs matured in both the reference and the atrazine microcosms further supports the conclusion that the occurrence of testicular oocytes is not related to atrazine exposure but rather a natural ontogenetic process. However, other endocrine modulators may have these effects. The frequency of testicular oocytes was increased in *X. laevis* (*14*, *15*) and *R. clamitans* (*17*) exposed to estradiol in the laboratory. The reason for the increase in the numbers of oocytes is not known but could be because the estradiol protects the oocytes from processes that result in their disappearance. Whether other endocrine modulators have similar effects is not yet known, but chronic exposures to bisphenol A at concentrations up to 500 μg/L were reported to not increase the frequency of testicular anomalies in developing *X. laevis* larvae (*10*).

Acknowledgments

Supporting Information Available

Table listing elements, as detected in water and sediment scans from the different microcosms (mg/L). This material is available via the Internet at http://pubs.acs.org.

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