ELSEVIER

Reproduction, larval growth, and reproductive development in African clawed frogs (*Xenopus laevis*) exposed to atrazine

Louis H. Du Preez [a,*], Nisile Kunene [a], Gideon J. Everson [a], James A. Carr [b], John P. Giesy [c,d,e], Timothy S. Gross [f], Alan J. Hosmer [g], Ronald J. Kendall [h], Ernest E. Smith [h], Keith R. Solomon [i], Glen J. Van Der Kraak [j]

[a] *School of Environmental Sciences and Development, North West University, Private Bag X6001, Potchefstroom 2520, South Africa*
[b] *Department of Biological Sciences, Texas Tech University, Lubbock, USA*
[c] *Toxicology Centre, University of Saskatchewan, Saskatoon, Saskatchewan, Canada*
[d] *Department of Biology and Chemistry, City University of Hong Kong, Tat Chee Avenue, Kowloon, Hong Kong, SAR, China*
[e] *Zoology Department, Michigan State University, East Lansing, MI, USA*
[f] *University of Florida, Gainesville, USA*
[g] *Syngenta Crop Protection, Greensboro, NC, USA*
[h] *The Institute of Environmental and Human Health and Department of Environmental Toxicology, Texas Tech University, Lubbock, USA*
[i] *Centre for Toxicology, University of Guelph, Guelph, ON, Canada*
[j] *Department of Integrative Biology, University of Guelph, Guelph, ON, Canada*

Abstract

Reproductive success and development of F2 offspring from F1 adult African clawed frogs (*Xenopus laevis*) exposed to atrazine throughout larval development and as sexually mature adults was examined. Larval *X. laevis* were exposed to one of four nominal concentrations of atrazine (0, 1, 10, 25 μg atrazine/l) beginning 96 hr after fertilization and continuing through two years post-metamorphosis. Clutch size and survival of offspring were used as measurement endpoints to gauge reproductive success of the F1 frogs. Larval survivorship and time to metamorphosis were used to gauge developmental success of the F2 offspring from atrazine-exposed frogs. Testes in F1 and F2 frogs were examined for incidence of anomalies, such as testicular ovarian follicles, and sex ratios in F2 offspring were investigated to determine if exposure to atrazine caused trans-generational effects (effects on F2 individuals due to exposure of F1 individuals). There were no effects of any of the studied concentrations of atrazine on clutch size of F1 frogs. There were also no effects on hatching success or time to metamorphosis. Sex ratios did not differ between F2 offspring among treatments. There was no evidence to suggest a transgenerational effect of atrazine on spawning success or reproductive development of *X. laevis*. This is consistent with the presence of robust populations of *X. laevis* in areas where they are exposed to atrazine that has been used for several decades for weed control in production of corn. Our observations also are consistent with the results of most other studies of frogs where no effects were found to be associated with exposure to atrazine. Our data do not support the hypothesis that atrazine significantly affects reproductive fitness and development of frogs.
© 2007 Elsevier Ltd. All rights reserved.

Keywords: Xenopus laevis; Atrazine; Testicular ovarian follicles; Transgenerational effects

1. Introduction

The potential effects of atrazine (CAS # 1912-24-9) on organisms in freshwater aquatic systems have been extensively reviewed (Solomon et al., 1996; Giddings et al.,

2005). Although some of the measurement endpoints used in these assessments, such as those in full life cycle studies on several species of fish and those in microcosms, include the aggregate responses of many possible mechanisms of action, these have not specifically characterized reproduction, the endocrine system, or development as possible targets for atrazine. These reviews noted the lack of data on some non-target terrestrial, aquatic, and semi-aquatic species such as reptiles and amphibians, especially for the newly identified sublethal endpoints related to development and reproduction (Solomon et al., 1996; Giddings et al., 2005).

A number of studies have been conducted in which the potential effects of atrazine on semi-aquatic species have been reported (see Solomon et al., 2005), including several laboratory studies investigating effects of atrazine on reproductive development in frogs. Some studies have reported that atrazine exposure during larval development results in the formation of hermaphroditic gonads and unpigmented ovaries at concentrations as small as 0.1 µg atrazine/l (Hayes et al., 2006) whereas others have found no effects on sex ratio or the incidence of testicular ovarian follicles (TOFs, the more correct term for testicular oocytes – see Hecker et al., 2006 for a review of the terminology) in African clawed frogs (*Xenopus laevis*) (Coady et al., 2004) and Green frogs (*Rana clamitans*) (Coady et al., 2005) or observed sexually ambiguous gonads only at greater concentrations (25 µg/l) of atrazine (Carr et al., 2003). The effects of atrazine on gonadal and kidney development in male and female *X. laevis* tadpoles were reported in a thesis and two published papers (Tavera-Mendoza, 2001; Tavera-Mendoza et al., 2002a,b). Unfortunately, the data in the published papers and in the thesis are inconsistent. The findings published in the literature were different than those in the thesis, from which the data presumably came and could not be repeated in a second study (Tavera-Mendoza, 2001). For these reasons, these data can not be interpreted as a reliable finding. A study by Hayes et al. (2002) was the first to report that atrazine concentrations, ranging from 0.1 to 200 µg atrazine/l produced gonadal abnormalities in developing *X. laevis* exposed from hatching until completion of metamorphosis. These authors reported that 16–20% of the exposed animals had multiple gonads or were 'hermaphrodites' with multiple testes and ovaries. There was no apparent association between atrazine concentration and the incidence of hermaphroditism or single sex polygonadism (Hayes, 2004). In a laboratory study, Hayes et al. (2003) exposed larval *R. pipiens* to 0.1 µg atrazine/l or 25 µg atrazine/l throughout larval development. They reported the presence of TOFs in males from the 0.1 and 25 µg atrazine/l treatment groups but not in the controls, although two control animals with oocytes associated with the testis were eliminated from the analysis (Hayes et al., 2003, p. 570).

Few studies have examined the effects of atrazine on frog development under natural conditions. A study by Hayes et al. (2003) reported that only frogs collected from sites with measurable atrazine concentrations developed TOFs and these were similar to those observed in frogs exposed under laboratory conditions to atrazine during their larval development. However, concentrations of atrazine were only measured in field water samples taken at the time of frog collection and there was no knowledge of atrazine concentrations that the frogs may have been exposed to prior to their collection – when they were undergoing gonadal differentiation. Furthermore, the effect was not observed in subsequent years (Hayes, 2005), even though adult frogs were present. This suggests that the effects, if related to atrazine at all, were transient. Studies in wild populations of *X. laevis* in areas of corn production and atrazine use (exposures from 0 to 9.3 µg/l) and in reference areas in South Africa revealed no effects on sex ratios of adults or metamorphs in relation to atrazine use (Du Preez et al., 2005a,b). In the same *X. laevis* populations, no differences in the absolute or relative numbers of testicular cell types were observed in *X. laevis* from corn and non-corn growing areas in South Africa (Smith et al., 2005). In studies on *X. laevis* larvae exposed to atrazine at concentrations up to 30 µg/l in outdoor microcosms, no effects on sex ratio were observed in stage 66 metamorphs (Jooste et al., 2005).

Despite equivocal results from laboratory and field studies, there are at present no published studies on the potential effects of atrazine on reproductive and offspring success. To assess the potential trans-generational effects (effects on F2 individuals due to exposure of F1 individuals) of atrazine on sexual development and reproduction, a multigenerational study was conducted in *X. laevis*. The study was a continuation of an earlier study where larvae were exposed to atrazine through to Nieuwkoop-Faber stage 66 in microcosms, the results of which were reported previously (Jooste et al., 2005). Specifically, various breeding crosses were conducted with frogs exposed to various concentrations of atrazine throughout their lives and effects on the numbers of eggs released, hatching success, and subsequent development of the F2 individuals was determined.

2. Methods and materials

2.1. Experimental design

Juvenile *X. laevis* that had been exposed throughout larval development in microcosms to nominal concentrations of 0, 1, 10 and 25 µg/l atrazine (Jooste et al., 2005) were used. The frogs were removed from the microcosm ponds upon completion of metamorphosis and individuals were freeze-branded with a number corresponding to the number of the microcosm pond in which they had been exposed to atrazine. Seventy-five frogs per atrazine concentration were randomly selected from each subset and transferred to 2,500 L tanks in a wet laboratory where they were continued to be exposed to the same concentrations of atrazine (in dechlorinated tap water) as in the microcosms from

which they originated. Photoperiod was from outdoor light. After the colder winter months, frogs were moved to 2,500 L outdoor pools containing atrazine at 0, 1, 10, and 25 μg/l, respectively. Frogs were fed three times a week on sinking *Xenopus* pellets (Avi Products, Durban RSA, based on the nutritional formula of the NASCO *Xenopus* frog brittle) and each container received 150 ml of pellets. All procedures involving *X. laevis* were approved by the Animal Care and Use Committee of the Northwest University (Ethical Clearance 03D14).

2.1.1. Treatments

A stock atrazine solution was prepared by dissolving 100 mg of technical active ingredient (CAS # 1912-24-9) in 1.0 L of analytical grade methanol as recommended for the FETAX assay (Interagency Coordinating Committee on Validation of Alternative Methods, 2000). This stock was diluted with water to achieve the required nominal concentrations and the total water volume was replaced with a fresh treatment solution on a monthly basis. Maximum concentration of methanol was 0.25 ml/l. In previous work using the same water sources and exposure conditions (Jooste et al., 2005), atrazine was shown to be relatively persistent with small concentration changes related to evaporation and rainfall over the 80-day exposure period. Concentrations of atrazine in the treatment solutions used in this component of the study were verified on four occasions using the analytical methods described previously (Du Preez et al., 2005b; Jooste et al., 2005). Mean concentrations (±SE) were 1.10 ± 0.11, 10.4 ± 0.29, and 24.8 ± 0.16 for the nominal 1, 10, and 25 μg atrazine/l exposures, respectively and <0.1 for the unexposed reference frogs.

2.1.2. Development and breeding

To allow easier observation of their development, after one year, frogs were transferred to 30 l glass aquaria where they were exposed to the same concentrations of atrazine. Stocking density was six frogs per tank. To ensure that they were all sexually mature, F1 frogs were allowed to grow in the respective atrazine solutions for a total of 2 yr. Mature males and females that had been reared in each concentration of atrazine were randomly selected for crossbreeding to assess reproductive success. In one set of crosses, a male from each treatment group was bred with a female from the reference group. In another cross, a male from the 25 μg atrazine/l group was bred with a female from the 25 μg atrazine/l group. Each combination was repeated with four pairs of frogs (Fig. 1). Spawning (in dechlorinated tap water) was induced by injecting the frogs with chorionic gonadotropin (Pregnyl, Donmed Pharmaceuticals (Pty) Ltd, Bedfordview, RSA) as described previously (Jooste et al., 2005). The first attempt at cross-breeding in November 2004 failed as the majority of the females released their eggs before the males were added to the spawning tanks. The breeding study was repeated in December with additional F1 frogs.
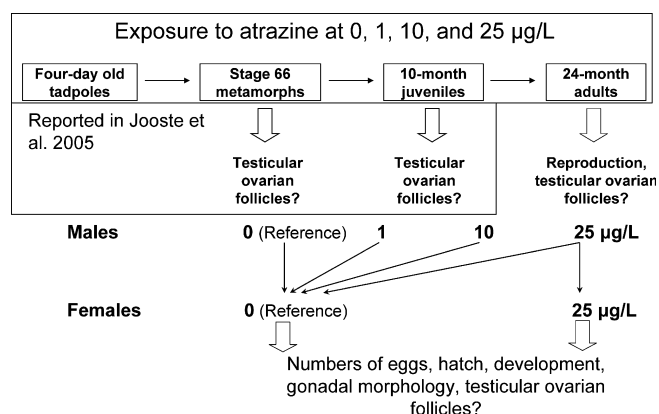

Fig. 1. Illustration of the experimental design and cross breeding.

2.1.3. F2 generation

Clutch size was determined by counting total number of eggs released by individual female frogs. Eggs were counted manually while syphoning them from a white tray (600×400 mm) into a second container. Hatching success within each clutch was determined by counting the number of three-day-old tadpoles in a shallow white tray (600×400 mm) containing water to a depth of 2 cm. This was done from 11 M pixel photographs (Nikon Coolpix 540 digital camera) imported into PowerPoint and overlaid with a 15×12 grid to aid in counting. Subsets of 50 tadpoles were randomly selected from each pairing (200 larvae total) and grown out to stage 66 in 30 L glass aquaria containing 25 L of constantly aerated FETAX medium (Dawson and Bantle, 1987) only (no atrazine exposure). Tadpoles were maintained in FETAX medium and fed every second day with *Xenopus* pellets that were soaked in water and then blended in a food processor. Tanks were cleaned 24 h after feeding. Larvae were checked daily to identify developing metamorphs. Tadpoles were counted weekly to determine survival.

2.1.4. Metamorphs

As tadpoles completed metamorphosis (Nieuwkoop-Faber stage 66), they were removed from the tanks, anesthetized using a 0.1% solution of 3-amino benzoic acid ethyl ester (MS 222), and body-mass measured using a Sartorius BP210S scale (0.0001 g accuracy). A small incision was made in the abdomen of each animal to allow penetration of the fixative and a tag with the date and identification number was attached to the right hind leg of the frog. Specimens were fixed in Bouin's for 48 h, rinsed in water, and then transferred to 70% ethanol. All the frogs were then dissected to expose the gonads for gross morphology. Gonadal phenotype was identified for each frog and the gonads digitally photographed using a Nikon Coolpix 900 digital camera fitted on a Nikon SMX1500 dissecting microscope, measured, and examined externally. Gonads of a randomly selected subset of 40 F2 frogs from each breeding cross were removed for histological examination.

2.1.5. Morphology and histology

Preserved testicular tissue was dehydrated in graded alcohols, embedded in paraffin wax, and serially sectioned longitudinally at 7 μm thickness using a Reichert Jung 2050 microtome (Jooste et al., 2005). Sections were stained with Harris haematoxylin and eosin and permanently mounted in DPX mounting medium (Saarchem Pty Ltd., Krugersdorp, South Africa). Sections were examined using Nikon Alphaphot compound microscopes and number of TOFs (Hecker et al., 2006) enumerated.

F1 male frogs used in cross breeding experiments were anesthetized with MS222, weighed, measured, examined externally. After a low-power inspection and photography of gonads using a Nikon Coolpix digital camera attached to a Nikon SMZ1500 dissecting microscope, one testis was fixed in Bouins for serial sectioning as above and histological examination as above. Statistics were conducted using SigmaStat (SPSS Science, 2004).

3. Results

3.1. Reproduction and hatching

Although females were randomly selected, size of females in the various parings differed and this resulted in large differences in number of eggs released. For example, one female in the Reference–Reference (0–0) group released 6717 eggs. Mean clutch size for the 0–0 group was 4165. Because, in general, clutch size was proportional to body mass (Fig. 2), the number of eggs released was corrected for the mass of the females (Fig. 3a). There were no significant differences in the number of eggs released per g of F1 body weight between groups ($p = 0.054$, ANOVA) and there was no indication of a concentration response, apart from the greater number of eggs released in the 0–0 crossing.

Hatching (Fig. 3b) varied within groups with no correlation between breeding combinations and hatching success. For example, hatching success varied from 36% to 96% in the 0–0 and 23–99% in the 25–0 combination. No concentration-response was observed. Time for the first larvae to reach NF stage 66 was similar across all groups (Fig. 3c) as
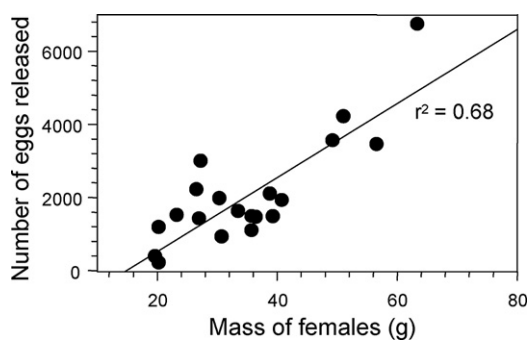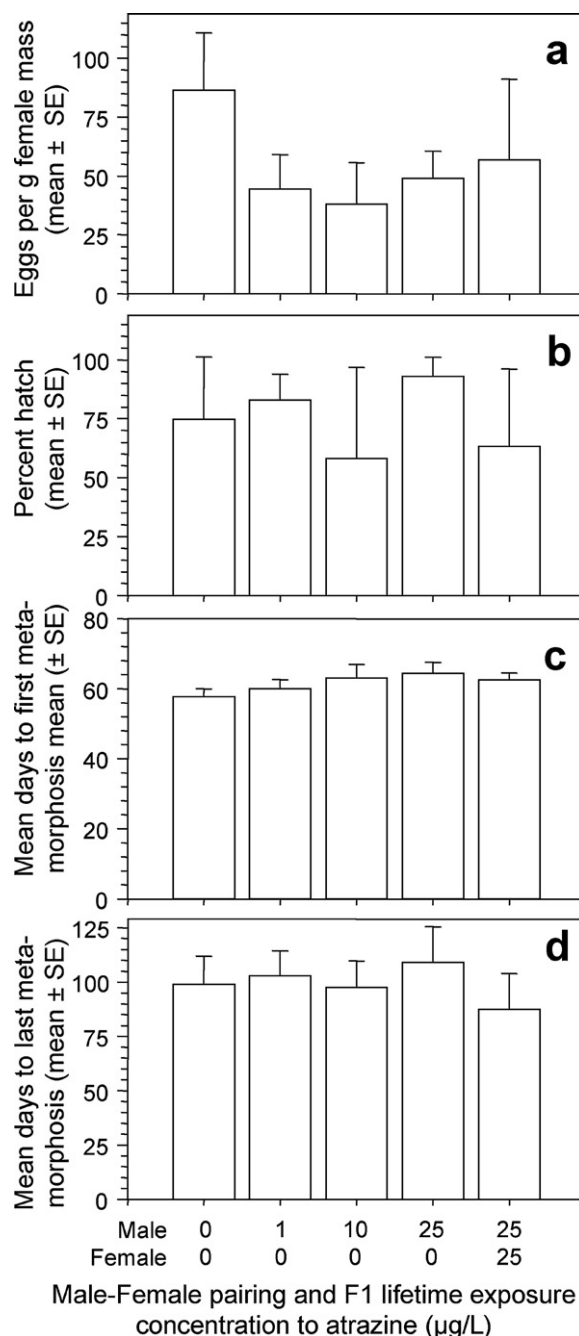

Fig. 3. a. Mass-corrected number of eggs released per F1 female. b. Percent hatch of F2 eggs. c. Days to reach stage 66 of the first F2 larva. d. Days to reach stage 66 of the last F2 larva. N = 4 pairs per treatment.

was the time for the last larva to reach NF stage 66 (Fig. 3d). No concentration-response was observed. These values were less than those observed for the F1 metamorphs in microcosms (70 d to first NF stage 66 metamorph). This was possibly because of the lower temperatures of the outdoor microcosms (Jooste et al., 2005).

Percent survival of the F2 larvae was similar in all pairings, except for the 1–0 pairing (Fig. 4a), which exhibited significantly less survival (ANOVA p value = 0.002), but no concentration-response was observed. For all the
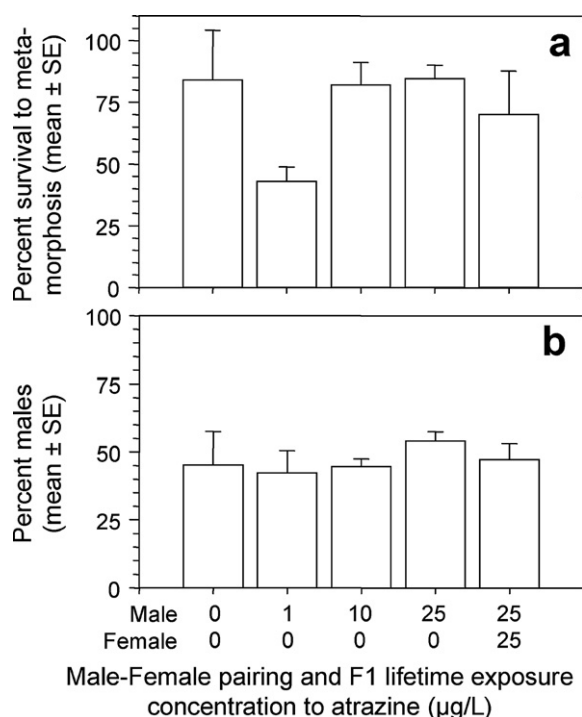

Fig. 2. Relationship between mass of F1 female frogs and number of eggs released.

Fig. 4. a. Survival of F2 larvae at stage 66. b. Percent males. Percent survival based on N = 200, sex-ratio on number of survivors.
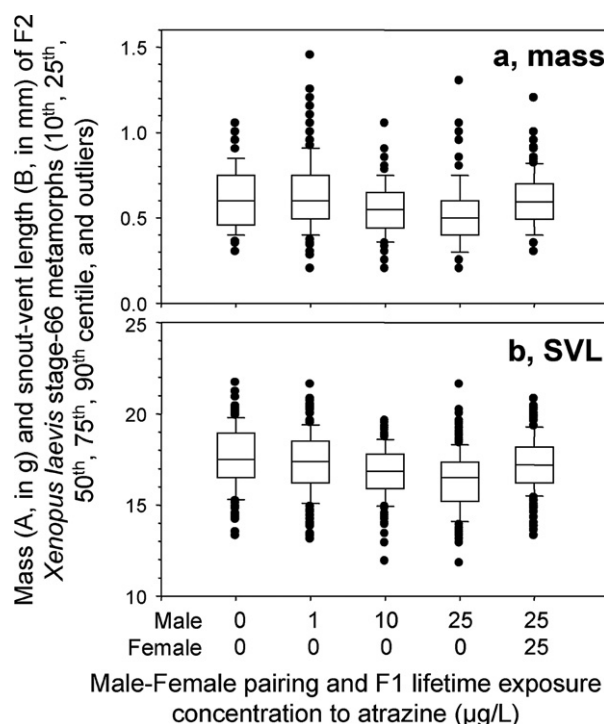

Fig. 5. a. Mass of F2 larvae at stage 66. b. Snout-Vent Length (SVL) of F2 larvae at stage 66. As data were not normally distributed, median, centiles, and outliers are show in a box-plot format. Data are the combined values for all the F2 progeny from the four pairs of adults and N ranged from 140 to 174.

groups except the 25–0 pairings, there were slightly more females than males (Fig. 4b).

Analysis of variance (AVOVA) of the median values for mass and snout-vent length (SVL) for progeny of each of the four frogs in the pairings gave p-values of 0.395 and 0.166 for mass and SVL, respectively, indicating no significant differences in weight or SVL of the F2 frogs. Based on the median values (Fig. 5), there appeared to be a concentration-related decrease in mass from male exposures to 1, 10, and 25 µg atrazine/l, however, the mass and SVL of the F2 frogs from the pairings where both the male and female frogs were exposed to 25 µg/l were similar to the reference pairings. Linear regression of the exposure concentrations for the pairs and the median mass and SVL of the F2 frogs gave r^2s of 0.107 and 0.16, respectively, suggesting no significant trend.

3.2. Testicular morphology and testicular ovarian follicles in F2 males

The only morphological anomalies observed in the testes of the F2 frogs were the presence of segmented testes (see Hecker et al., 2006) and the complete absence of one of the testes. Segmented testes were observed in two of 76 males (2.6%) from the 0–0 combination, one of 74 males (1.4%) of the 1–0 combination, one of 94 (1%) of the 10–0 combination, and zero of 91 and 110 (0%) of the 25–0 and 25–25 pairings, respectively. Single testes were observed in two (2.6%) of the 0–0 pairing, one (1.4%) of the 1–0, one (1%) of the 10–0 combination and zero (0%) of the 25–0 and 25–25 combinations, respectively.

A low frequency of TOFs was observed for all combinations of pairings with considerable variability among pairings (Fig 6a). The incidence of TOFs in F2 frogs ranged from 5% to 15%. There was no indication of a concentration-response relationship in either the frequency of frogs with TOFs (Fig. 6a) or the number of oocytes per frog (Fig. 6b). There was no significant difference between treatments in the number TOFs per testis in the F2 frogs (Kruskal-Wallis One Way Analysis of Variance on Ranks, $p = 0.071$).

3.3. Testicular Morphology and Testicular Ovarian Follicles in F1 Males

Histological screening of the testes of the F1 adults after breeding, revealed only one TOF, in one of the 25-R F1 males. Earlier observations in the 10-month old F1 males showed a greater incidence of TOFs (Jooste et al., 2005) than observed in the 2 yr old F1 males.

4. Discussion and conclusions

Exposure of *X. laevis* to nominal concentrations of atrazine of 1, 10, and 25 µg atrazine/l from age 96 h to 2 yrs had no effect on reproduction in the F1 generation and a number of indicators of reproductive success in the F2 larvae. There were no indications of responses to concentration of atrazine in terms of the development of the F1
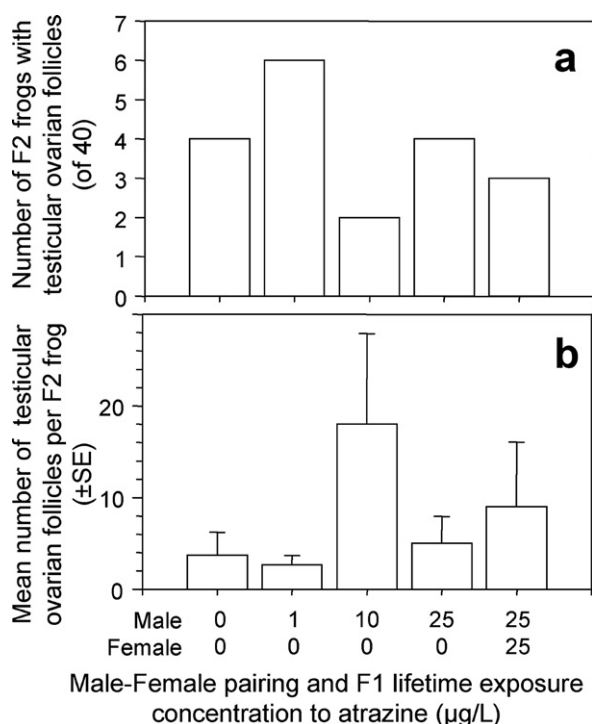
Fig. 6. a. Number of F2 frogs with testicular ovarian follicles. b. Number of testicular ovarian follicles in those F2 frogs with ovarian follicles in one or both testes. N ranged from 74 to 110.

frogs through to the juvenile stage (Jooste et al., 2005) or in the reproduction of the F1 adults. Likewise, there was no concentration response between atrazine exposures and hatchability, time to metamorphosis or body size at metamorphosis as determined by body mass and snout-vent length, suggesting that the offspring of atrazine exposed frogs are not at a developmental disadvantage (Wilbur and Collins, 1973). This is also consistent with the presence of robust population of frogs in association with atrazine use and crop production (Hayes et al., 2003; Knutson et al., 2004; Du Preez et al., 2005b).

The incidence of TOFs in F2 frogs and the number of TOFs per testis were both less than those observed in a sub-sample of the F1 generation metamorphs (Jooste et al., 2005). The F2 larvae in this study were raised under laboratory conditions with aeration, regular replacement of FETAX medium, and abundant food. The F1 larvae were raised to metamorphs in microcosms under semi-field conditions where they were exposed to greater variation in temperature, experienced changes in oxygen concentration, and may have experienced more competition for food resources. These additional stressors under field conditions may have influenced the incidence and frequency of TOFs.

Histological screening of the testes of the adults after breeding revealed that only one F1 frog (from the 25–0 pairings) had a testis containing a single TOF. None of the other frogs sampled contained TOFs. Although the number of adults screened was small (4) this is consistent with regression of the follicles (see definition in Hecker

et al., 2006) over time as was suggested in observations of the F1 generation (Jooste et al., 2005).

There were no exposure-related trends in the incidence of F2 frogs with TOFs and the number of TOFs in these frogs. This observation is consistent with many other laboratory and field studies in *X. laevis* (Coady et al., 2005; Du Preez et al., 2005b; Hecker et al., 2004,2005a,b; Smith et al., 2005) and other frogs (Coady et al., 2004; Reeder et al., 2005). There was no evidence to suggest any transgenerational effects of atrazine on spawning success or reproductive development of *X. laevis*. This is consistent with the presence of robust populations of *X. laevis* in areas where exposures to atrazine have been observed (Du Preez et al., 2005b) and where it has been used in corn production for several decades.

Considering the results of this study, as well as a large majority of other studies that have not observed effects of atrazine on a number of reproductive endpoints in *X. laevis* and other frogs (see above references), there is little evidence to support the theory that exposure to environmentally relevant concentrations of atrazine adversely affects reproduction and reproductive development in *X. laevis*.

Acknowledgements

References

Carr, J.A., Gentles, A., Smith, E.E., Goleman, W.L., Urquidi, L.J., Thuett, K., Kendall, R.J., Giesy, J.P., Gross, T.S., Solomon, K.R., Van Der Kraak, G.J., 2003. Response of larval *Xenopus laevis* to atrazine: assessment of gonadal and laryngeal morphology. Environ. Toxicol. Chem. 22, 396–405.

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

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

Dawson, D.A., Bantle, J.A., 1987. Development of a reconstituted water medium and preliminary validation of the frog embryo teratogenesis assay – *Xenopus* (FETAX). J. Appl. Toxicol. 7, 237–244.

Du Preez, L.H., Jansen van Rensburg, P.J., Jooste, A.M., Carr, J.A., Giesy, J.P., Gross, T.S., Kendall, R.J., Smith, E.E., Van Der Kraak, G., Solomon, K.R., 2005a. Seasonal exposures to triazine and other pesticides in surface waters in the western Highveld corn-production region in South Africa. Environ. Pollut. 135, 131–141.

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.J., Weldon, C., 2005b. Population structure of the African clawed frog (*Xenopus laevis*) in maize-growing areas with atrazine application versus non-maize-growing areas in South Africa. Afr. J. Herp. 54, 61–68.

Giddings, J.M., Anderson, T.A., Hall Jr., L.W., Kendall, R.J., Richards, R.P., Solomon, K.R., Williams, W.M., 2005. A Probabilistic Aquatic Ecological Risk Assessment of Atrazine in North American Surface Waters. SETAC Press, Pensacola, FL, USA.

Hayes, T.B., 2004. This is no denying this: defusing the confusion about atrazine. Bioscience 54, 1138–1149.

Hayes, T.B., 2005. Atrazine and pesticide mixtures: sum of the parts or some of the parts? SETAC Annual Meeting, Baltimore, MD, USA, SETAC, Pensacola, FL.

Hayes, T.B., Collins, A., Mendoza, M., Noriega, N., Stuart, A.A., Vonk, A., 2002. Hermaphroditic, demasculinized frogs exposure to the herbicide atrazine at low ecologically relevant doses. Proc. Natl. Acad. Sci. USA 99, 5476–5480.

Hayes, T.B., Haston, K., Tsui, M., Hoang, A., Haeffele, C., Vonk, A., 2003. Atrazine-induced hermaphroditism at 0.1 ppb in American leopard frogs (*Rana pipiens*): laboratory and field evidence. Environ. Health Perspect. 111, 568–575.

Hayes, T.B., Stuart, A.A., Mendoza, M., Collins, A., Noriega, N., Vonk, A., Johnston, G., Liu, R., Kpodzo, D., 2006. 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. Environ. Health Perspect. 114 (Suppl. 1), 134–141.

Hecker, M., Giesy, J.P., Jones, P.D., Jooste, A.M., Carr, J.A., Solomon, K.R., Smith, E.E., Van Der Kraak, G.J., Kendall, R.J., Du Preez, L.H., 2004. Plasma sex steroid concentrations and gonadal aromatase activities in African clawed frogs (*Xenopus laevis*) from the corn-growing region of South Africa. Environ. Toxicol. Chem. 23, 1996–2007.

Hecker, M., Kim, W.J., Park, J.-W., Murphy, M.B., Villeneuve, D., Coady, K.K., Jones, P.D., Solomon, K.R., Van Der Kraak, G.J., Carr, J.A., Smith, E.E., du Preez, L.H., Kendall, R.J., Giesy, J.P., 2005a. Plasma concentrations of estradiol and testosterone, gonadal aromatase activity, and ultrastructure of the testis in *Xenopus laevis* exposed to estradiol and atrazine. Aquat. Toxicol. 72, 383–396.

Hecker, M., Murphy, M.B., Coady, K.K., Villeneuve, D.L., Jones, P.D., Carr, J.A., Solomon, K.R., Smith, E.E., Van Der Kraak, G.L., Gross, T.S., du Preez, L.H., Kendall, R.J., Giesy, J.P., 2006. Terminology of gonadal anomalies in fish and amphibians resulting from chemical exposures. Rev. Environ. Contam. Toxicol. 187, 103–132.

Hecker, M., Park, J.-W., Murphy, M.B., Jones, P.D., Solomon, K.R., Van Der Kraak, G.J., Carr, J.A., Smith, E.E., Du Preez, L.H., Kendall, R.J., Giesy, J.P., 2005b. Effects of atrazine on CYP19 gene expression and aromatase activity in testes and on sex steroid concentrations in plasma of male African clawed frogs (*Xenopus laevis*). Toxicol. Sci. 86, 273–280.

Interagency Coordinating Committee on Validation of Alternative Methods, 2000. Background Review Document Frog Embryo Teratogenesis Assay – Xenopus (FETAX). National Institute of Environmental Health Sciences Research Triangle Park, NC, USA, National Institute of Environmental Health Sciences, Report Available from <http://iccvam.niehs.nih.gov/methods/fetaxdoc/fetaxbrd.htm> p. 92.

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

Knutson, M.G., Richardson, W.B., Reineke, D.M., Gray, B.R., Parmelee, J.R., Weick, S.E., 2004. Agricultural ponds support amphibian populations. Ecol. Appl. 14, 669–684.

Reeder, A.L., Ruiz, M.O., Pessier, A., Brown, L.E., Levengood, J.M., Phillips, C.A., Wheeler, M.B., Warner, R.E., Beasley, V.R., 2005. Intersexuality and the cricket frog decline: historic and geographic trends. Environ. Health Perspect. 113, 261–265.

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

Solomon, K.R., Baker, D.B., Richards, P., Dixon, K.R., Klaine, S.J., La Point, T.W., Kendall, R.J., Giddings, J.M., Giesy, J.P., Hall, L.W.J., Weisskopf, C., Williams, M., 1996. Ecological risk assessment of atrazine in North American surface waters. Environ. Toxicol. Chem. 15, 31–76.

Solomon, K.R., Carr, J.A., du Preez, L.H., Giesy, J.P., Gross, T.S., Kendall, R.J., Smith, E.E., Van Der Kraak, G.J., 2005. Ecotoxicological risk assessment of atrazine in amphibians. In: Clark, J.M., Ohkawa, H. (Eds.), Environmental Fate and Safety Management of Agrochemical. ACS Symposium Series No. 899. vol. 2 American Chemical Society, Washington, DC, USA, pp. 124–137.

SPSS Science, 2004. SigmaStat for Windows. SPSS Science, Chicago, IL, USA.

Tavera-Mendoza, L., Ruby, S., Brousseau, P., Fourier, M., Cyr, D., Marcogliese, D., 2002a. Response of the amphibian tadpole (*Xenopus laevis*) to atrazine during sexual differentiation of the testis. Environ. Toxicol. Chem. 21, 527–531.

Tavera-Mendoza, L., Ruby, S., Brousseau, P., Fourier, M., Cyr, D., Marcogliese, D., 2002b. Response of the amphibian tadpole *Xenopus laevis* to atrazine during sexual differentiation of the ovary. Environ. Toxicol. Chem. 21, 1264–1267.

Tavera-Mendoza, L.E., 2001. Influences of atrazine on gonadal differentiation in *Xenopus laevis* tadpoles during metamorphosis. M.Sc thesis. Department of Biology. Concordia University, Montreal, PQ, Canada, p. 73.

Wilbur, H.M., Collins, J.P., 1973. Ecological aspects of amphibian metamorphosis. Science 182, 1305–1314.