Characterisation of gonadal responses in *Xenopus laevis* to exposures of atrazine in semi-natural microcosms

N. KUNENE

12925179

Dissertation submitted in partial fulfilment of the requirements for the degree Magister in Environmental Sciences at the North-West University, Potchefstroom Campus

Supervisor: Prof L.H. Du Preez

December 2008
Potchefstroom

NORTH-WEST UNIVERSITY
YUNIBESITI YA BOKONE-BOPHIRIMA
NOORDWES-UNIVERSITEIT

Acknowledgements

I would like to express my greatest appreciation to God who gave me the strength to complete this study.

My gratitude also extends to Prof. L.H. Du Preez, who motivated and guided me throughout this study. Thanks to his constant support which was always given with enthusiasm.

Thank you to Dr. C. Weldon for his support and assistance through all the stages of this study as well as other colleagues at Zoology.

I am also grateful to the following people:

My husband, Bongani Kunene, who gave me all the encouragement and support that I needed.

My children Senamile, Lotive, Tenele, Nonceba and Mhlo who encouraged and motivated me.

My mother, Princess Ngebeti, and my sister Lomantjolo, as well as my brothers and my father in law A.V.Kunene who urged me not to give up.

Abstract

The African Clawed frog (*Xenopus laevis*) is most likely the most studied amphibian to date. This animal is widely used as a laboratory screening model for the testing of various chemicals. In recent years, there has been considerable controversy over the possible effects of the widely-used herbicide atrazine on amphibians. There were claims that this broadleaf herbicide causes gonadal abnormalities in amphibians, including feminisation and the promotion of a form of abnormality in the testes characterised by the presence of ovarian follicles (oocytes). Clawed frogs are native to Africa and were used in this study to test the reproductive success and development of F2 offspring after the F1 parent animals were exposed to known atrazine concentrations from 96 hrs to 24 month-old mature frogs. Animals were exposed to four nominal concentrations of atrazine (0, 1, 10, 25 µg/ℓ). Male and female frogs were paired off according to the atrazine concentration in which they were reared and spawning was induced. Clutch size and survival of offspring were used to evaluate developmental success. Gonads of metamorphs as well as breeding F1 frogs were examined for gross anomalies. Testes were serially sectioned and screened for anomalies at the microscopic level. We were unable to find any concentration response to hatching success, time to metamorphosis or sex ratios. No indication of a transgenerational effect of atrazine on spawning success or reproductive development of *X. laevis* was observed. Adult *X. laevis* collected along a north-south transect from the south-west Western Cape region to the north-east were analysed and screened for gonadal anomalies. We found that, irrespective of exposure to atrazine, male *X. laevis* from north-east sites contained testicular ovarian follicles whereas none of the animals from the Western Cape sites had any. Differences between these populations of *X. laevis* have been reported and it cannot be excluded that they belong to separate species, in which case the phenomenon of testicular ovarian follicles could be associated with the northern form and with no relevance to atrazine usage. Atrazine has been widely used in South Africa for more than 40 years, and still robust populations of *X. laevis* with balanced sex ratios occur throughout its distribution range - which include the major maize production area in South Africa. Our data does not support the hypothesis that atrazine impacts negatively on amphibians in natural situations and at environmentally relevant concentrations.

Opsomming

Die Gewone platanna (*Xenopus laevis*) is wêreldwyd waarskynlik die mees bestudeerde paddaspesie en word algemeen gebruik in die evaluering van verskeie chemiese middele. Daar was onlangs 'n groot mate van kontroversie oor die moontlike skadelike effek wat 'n algemeen-gebruikte breëblaar onkruiddoder, atrasien, op amfibiërs mag hê. Daar is beweer dat dié middel lei tot die ontwikkeling van abnormale gonades asook tot die vervrouliking van manlike paddas, en dat dit ook aanleiding gee tot die vorming van testes met ingeslote ovariumfollikels (oösiete). Platannas is endemies aan Afrika en is in hierdie studie gebruik om die voortplantingsukses en ontwikkeling van die F2-generasie te toets nadat die F1-ouers blootgestel is aan bekende konsentrasies atrasien. Die F1-paddas is blootgestel vanaf 96 uur-oue paddavisse tot twee jaar-oue volwasse paddas. Proefdiere is blootgestel aan nominale konsentrasies atrasien (0, 1, 10, 25 µg/ℓ). Manlike en vroulike paddas is afgepaar volgens 'n blootstellingsmodel en geïnduseerde bevrugting is toegepas. Die aantal eiers wat gelê is sowel as oorlewing is gebruik as aanduiding van die sukses van die ontwikkeling. Gonades van jong paddatjies wat metamorfose voltooi het, asook dié van die broeipare is ondersoek op makroskopiese asook mikroskopiese vlak. Seriesneë is deur testes gemaak en mikroskopies ondersoek. Geen verwantskap tussen atrasienkonsentrasie en die voorkoms van afwykings kon gevind word nie en uitbroeisukses, tydsduur na voltooiing van metamorfose asook die geslagsverhouding het normaal voorgekom. Verder is geen aanduiding gevind dat die nageslag van blootgestelde paddas enigsins benadeel is nie. Volwasse *X. laevis* wat langs 'n noord-suid transek, vanaf die suid-westelike Weskaapse omgewing tot en met die noord-ooste versamel is,, is bestudeer vir moontlike gonadale afwykings wat in verband gebring sou kon word met die gebruik van pestisiede. Daar is gevind dat, ongeag die voorkoms van pestisiede, testikulêre ovarium follikels algemeen voorkom in paddas wat versamel is in die noord-oostelike versamelpunte terwyl geen gevind is in die Weskaap nie. Verskille tussen die populasies platannas is al gerapporteer en aanduidings bestaan dat dit moontlik twee verskillende spesies kan wees. Die voorkoms van die follikels in testes het waarskynlik weinig te make met enige stowwe in die omgewing nie, maar is 'n verskynsel wat by die noordelike vorm aangetref word. Atrasien word al vir meer as 40 jaar op groot skaal in Suid-Afrika toegedien en robuuste populasies platannas met gebalanseerde verhouding tussen mannetjies en wyfies kom steeds regdeur sy verspreidingsgebied voor, insluitende die area waar die meeste mielies verbou word. Ons data ondersteun dus nie die hipotese dat atrasien negatief impakteer op platannas in natuurlike toestande nie.

Table of Contents

Chapter 1

Introduction and Literature Overview

Chapter 1

Introduction and Literature Overview

The global decline in amphibian populations is one of the most vexing conservation issues of recent times. Because amphibians frequently have complex life histories and occupy multiple niches throughout their lives, they are important components of many aquatic and terrestrial ecosystems. Single amphibian species can play multiple ecological roles as aquatic consumers and terrestrial predators, and amphibians are an important prey for a number of vertebrate taxa and some arthropod larvae, acting as an important link among trophic levels (Burton & Likens, 1975). The ecological importance of amphibians in both terrestrial and aquatic ecosystems suggests that the loss of members of this group will have complex and wide-ranging consequences. It is therefore particularly troubling that amphibians are now the most-threatened class of vertebrates. A recent report from the IUCN's Global Amphibian Assessment suggests that as many as a third of amphibian species (> 5 700) have undergone severe declines or extinction with over 7% listed as critically endangered and many species on the brink of extinction (IUCN Red List Data; Stuart *et al.*, 2004). Despite increased scientific awareness of the threats facing amphibians, the recent increase in amphibian extinctions is largely unexplained, in part because many of these extinctions have occurred in virtually undisturbed tropical "refuges," and often montane areas isolated from the adverse effects of habitat destruction and pollution (Pounds *et al.*, 1997; Pounds & Crump 1994; Wyman, 1990; Wake, 1991). The first reports of amphibian declines were received with skepticism as seasonal fluctuation of amphibian population size is a natural occurrence. Although a number of viable hypotheses have been presented to explain such enigmatic extinctions and declines (e.g., Alford & Richards, 1999; Blaustein & Kiesecker, 2002; Collins & Storfer, 2003), scientific consensus on the causes of amphibian declines has been elusive, and synergisms among factors may be obfuscating the root mechanisms of amphibian declines (Blaustein *et al.*, 2003; Pounds *et al.*, 1999).

The identification of amphibian chytrid as a causal factor in numerous global declines supports amphibian chytrid as a potential link among global amphibian declines (Bell *et al.*, 2004; Lips *et al.*, 2004) and indicates that amphibian chytrid may be spread

by anthropogenic activities. Weldon (2002) also conducted a survey on chytridiomycosis as a cause of amphibian decline in South Africa. Habitat destruction further impacts heavily on amphibians. Another aspect that received a great deal of attention lately is the effect of agrocemicals. Various chemicals are believed to disrupt the normal hormonal systems of the body. Those that are known to have an effect include organochlorines, pesticides, triazines, pyrethoids and heavy metals such as Cd, Pb and Hg (Yu, 2000).

Recent studies in especially mammals and reptiles suggest that atrazine, a broad leaf herbicide, may interfere with the endocrine regulation of reproduction, possibly through effects at the level of the hypothalamus (Cooper *et al.*, 2000; Crain *et al.*, 1997; Sanderson *et al.*, 2000; Sanderson, 2001). In addition, several field surveys have linked amphibian malformities with pesticide use (Ouellet *et al.*, 1997). Atrazine is widely used in South Africa and especially so on the central highveld which is the major maize production area in South Africa, and where maize is usually planted during September/October. Maize is normally treated with atrazine and terbuthylazine in a 1:1 mixture at 600 g/ha of each active ingredient in one or two treatments in October and/or November to December with the total applied equal to 1 to 1.5 the recommended rate. A surfactant may be used in combination with this application and normally consists of a nonylphenol ethoxylate mixed with the atrazine-terbuthylazine at a rate of 125 to 250 g/ha. A number of insecticides such as the pyrethroids, endosulfan, monocrotophos and seed treatments may be used on maize and could be confounders in the study. In addition, metals may also be confounders.

The study of atrazine followed a phased approach. This study investigates the gonadal responses in *Xenopus laevis* to exposures of atrazine in semi-natural microcosms. The herbicide is investigated because it has been suggested to be a chemical that is an endocrine disrupter (Calborn, 1998). Atrazine is a triazine herbicide extensively used in maize production. It is one of the two most commonly used agricultural pesticide in the US or even in the world (U.S. EPA. 2001).

Atrazine is a colourless crystalline powder with low vapor pressure (40 nPa at 20°C) and a melting point range of 175 – 177 °C. It is readily soluble in dimethyl sulfoxide (183g/litre), slightly soluble in methanol (18g/litre), diethyl esther (12g/L), chloroform (52g/L) and ethyl acetate (28g/L) and very slightly soluble in water

(30mg/litre). It is stable in the dry state but is hydrolysed to the herbicidally inactive 2-hydroxy analogue in acid or in alkaline solutions and more slowly in neutral aqueous solutions. It has a relative molecular mass of 215.7g. The chemical formula for atrazine is $C_8H_{14}ClN_5$. Gas chromatography with a nitrogen-phosphorous detector (NDP) is generally used for the determination of residues and the analysis of environmental samples. The minimum detection limit varies according to the substrate.

Atrazine was introduced in 1958. In 1987, total worldwide production was estimated to be 70 000 tonnes (IPCS, 1990). It is a selective pre– and post-emergence herbicide which is used for the control of weeds in crops such as asparagus, maize, sorghum, sugar cane and pineapple (IPCS, 1990) with its largest market in maize production (Wicks, 1998). It is also used in forestry and, at higher application rates, for non-selective weed control in non-crop areas such as railways, roadsides and industrial areas.

Many amphibians species, especially frogs, complete their life-cycles in temporary breeding sites or shallow ponds which are near agricultural fields that receive pesticide application. Findings of similar effects on sexual development in two diverse species (*Xenopus laevis* and *Rana pipiens*) show that the effects of atrazine are not restricted to a single species and are, in fact, likely a problem for amphibians in general (Hayes, *et al.,* 2002).

Applied as a pre-emergent, atrazine contamination of water sources peaks with spring rains. The timing of atrazine contamination of water sources directly coincides with amphibian breeding activities, since many amphibians reproduce during early spring rains and thus the potential impact of atrazine on amphibians is significant. Many amphibian species are in decline (Wake, 1991; Blaustein *et al.*, 2002; Gardner, 2001) and *Rana pipiens* populations are also declining in many locations in Indiana and Illinois in the U.S.

Studies have documented effects of atrazine on amphibians at relatively low concentrations. A study conducted by scientists at the University of Mississippi found that concentrations of 20µg/L caused mortality of tadpoles of the frog *Hyla chrysoscelis* (Britson *et al.*, 2000). A USGS study of larval tiger salamanders found that 75µg/L of

atrazine caused blood levels of one growth hormone (thyroxin) to rise and another (corticosterone) to decrease. The result was that the salamanders' metamorphosis was slowed down (Larson *et al.*, 1998).

Hayes *et al.* (2000) showed that atrazine exposure of 0.1µg/L resulted in retarded gonadal development and testicular oogenesis (hermaphroditism) in leopard frogs (*Rana pipiens*) which is a U.S. native species. They found that slower developing males even experienced ovarian follicle growth (vitellogenesis). It was furthermore observed that there were gonadal dysgenesis (gonadal development) and hermaphroditism in animals collected from atrazine–contaminated sites across the U.S. Reeder *et al.* (1998) described testicular ovarian follicles in field–collected frogs (*Acris creptians*) and suggested that atrazine may be involved in this abnormality, but did not have laboratory data to support the suggestion.

In the nineteenth century European scientists discovered an unusual amphibian in the Cape Colony (South Africa). They called it "Le Crapand Lisse" (smooth – skinned frog) and named it *Xenopus* (strange foot) laevis (smooth) (Measey, 1998). The animal was already known by the people living in Sub-Saharan Africa as a protein source and as an aphrodisiac or medicine for fertility. X. *laevis* is a standard laboratory amphibian because it is easy to breed and maintain. Being aquatic throughout their lives, X. *laevis* are easy to keep and are resistant against disease and infection. The X. *laevis* was used as an assay for luteinising hormone and thus pregnancy testing (Measy, 1998). This African clawed frog has a wide distribution area within the boundaries of South Africa, occurring from the Western Cape Province northwards, excluding the extreme North of the Northern Cape Province, northern Kwa-Zulu Natal and eastern Mpumalanga (Weldon, 1999). Subsequent use of *X. laevis* as a laboratory amphibian in schools, universities, pregnancy clinics, medical research establishments and as pets has meant that this animal is familiar to biologists all over the world and has even established feral populations (Measey, 1998).

Tavera-Menduza *et al.* (2002a) showed that atrazine exposure (21µg/L) for as little as 48 hours resulted in severe gonadal dysgenesis in the African clawed frogs (*X. laevis*). It has been shown that atrazine induced hermaphroditism at concentrations of only 0.1µg/L (Hayes *et al.,* 2002) when administered throughout larval development.

Hayes *et al.* (2002) investigated the effects of *X. laevis* which were exposed, from hatching to metamorphosis, to atrazine concentrations ranging from 0.1 to 200 µg/L. They observed that there was no effect on the larval growth, developmental rate, mortality, time to metamorphosis or size at metamorphosis in females or males (Hayes *et al.* 2002) but they reported that atrazine treatment (0.1 – 25 µg/L) decreased laryngeal size in male but not female *X. laevis* relative to unexposed controls and also increased the incidence of gonadal abnormalities. However, Carr *et al.* (2003) also reported that atrazine concentrations up to 25 µg/L did not have any effect on male *X. laevis* laryngeal size and there was only a significant increase in gonadal abnormalities at 25 µg/L.

Carr *et al.* (2003) reported that exposure of *X. laevis* larvae from when they are 48 hrs or 72 hrs old to completion of metamorphosis (stage 66), to atrazine at concentrations of 1.10 and 25 µg/L, showed no effects on post-hatching of treatment groups compared to reference groups. In both the reference groups and experimental groups the hatching success was greater than 90% (Carr *et al.*, 2003). Based on the gonadal morphology of the animals in the 25µg/L exposure group, these were animals that showed intersex. Although the X. *laevis* in this group had gonads that were different in shape, size and pigmentation from the reference group, the histological evaluation revealed that most of the intersex *X. laevis* had gonads that could be identified as either male or female (Carr *et al.*, 2003). A similar study was conducted by Coady *et al.* (2003). They exposed post–metamorphic *X. laevis* to atrazine at concentrations of 0.1, 1, 10 and 25 µg/L. Based on the gross morphology of the gonads, atrazine did not cause any concentration-dependant effects on the gonad development or the frequency of gonadal anomalies (Coady *et al.*, +-2003).

Atrazine may not be the only compound that induces testicular oogenesis. There may be many chemicals, natural products and even populations that naturally display this phenomenon (Witschi, 1929). According to Wake (2004) although atrazine has been shown to be harmful in some instances, some people claim that it should not be banned without sound scientific proof that it is harmful in the environment. Male frogs with female characteristics have been documented since the 1920's, decades before the introduction of atrazine. Perhaps scientists are just beginning to realise how widespread this phenomena is in the wild. It is not necessarily true that atrazine began causing these

problems after over forty years of widespread use. Other factors may be at work. There are many species of frogs, such as the leopard frog, that are thriving in atrazine-contaminated areas (Wake, 2004).

The main objectives of this study are:

a) To determine whether exposure to atrazine would influence the reproductivity of *X. laevis.*

b) To determine whether the sex ratio of the F1 generation of *X. laevis* that has been exposed to atrazine differs from the control group.

c) To determine whether exposure to various concentrations of atrazine would cause any adverse effects on the gonads of *X. laevis.*

d) To determine whether gonadal anomalies show a dose response.

e) To determine whether doses of atrazine affect testicular ovarian follicles.

The second part of the study aims to determine the testicular ovarian follicle prevalence in *X. laevis* from areas which are free of atrazine in South Africa. The objectives of this part of the study are:

a) To determine whether testicular ovarian follicles is a natural phenomenon in *X. laevis.*

b) To determine whether the prevalence of testicular ovarian follicles could be linked to atrazine use.

c) To determine whether there are differences in the prevalence in testicular ovarian follicles in *X. laevis* strains from the Western Cape and from north of Cape fold mountains in South Africa.

d) To study the nature and variation in testicular ovarian follicles

e) To determine whether there is any relationship between the age of a frog and testicular ovarian follicle prevalence.

Chapter 2

Materials and Methods

Chapter 2

Materials and Methods

2.1 Cross breeding

2.1.1. Source of frogs

The frogs that were used in this study were reared by Alarik Jooste (Jooste, 2003) during his study to evaluate the effects of atrazine exposure on *X. laevis* in South Africa. In March 2002, Jooste constructed 12 microcosms outdoors at the experimental facility of the Potchefstroom Campus of the North-West University, South Africa. Each microcosm was 2.25 m long, 1.2 m wide and 1.0 m deep and was lined with a polythene membrane (fig. 2.1). Each held 1100 L of water. The water level was maintained throughout the study by adding tap water (Jooste, 2003). Macrophytes (ceratophylum) from field sites were introduced after which the microcosms were allowed to stabilise for five months.

In August 2002, the 12 microcosms were randomly allocated in three sets of four each. One set of three microcosms received no atrazine and served as reference. A stock solution of atrazine was prepared by dissolving 100 mg atrazine in 1L analytical grade methanol. Three microcosms per concentration were treated with atrazine to achieve initial concentrations of 1 µg/L, 10 µg/L and 25 µg/L respectively. At the end of 2002, the exposure study commenced.

Spawning was induced on the male and female frogs after which they were placed together in pairs in breeding tanks. Tadpoles hatched in two days and were then exposed to concentrations of 0, 1, 10 and 25 µg/L atrazine in the prepared microcosm ponds from when they were 96 hours of age up until the time when they have completed metamorphosis. 888 tadpoles were released into each microcosm. After metamorphosis, 75 frogs per concentration were selected from the subsets and transferred to 4 x 1 000 L grow–out tanks in a wet lab where they were exposed to the same concentrations of atrazine as in the microcosms from which they originated. After the winter, the F_1 generation frogs were transferred to 4 x 1 000L outdoor microcosm ponds containing the same concentrations of atrazine, namely 0, 1, 10 and 25 µg/L where they were kept for two years and fed ox heart twice a week.

Figure 2.1: Microcosms for tadpole rearing

2.1.2 Breeding combinations

For this study, mature males and females from Jooste's F1 generation were used.(Jooste,2003) A male from each treatment group was bred with a female from the reference group then a male from 25 µg/L group was bred with a female frog from the 25 µg/L group (fig. 2.2, 2.3). Each combination was performed with four pairs of frogs.

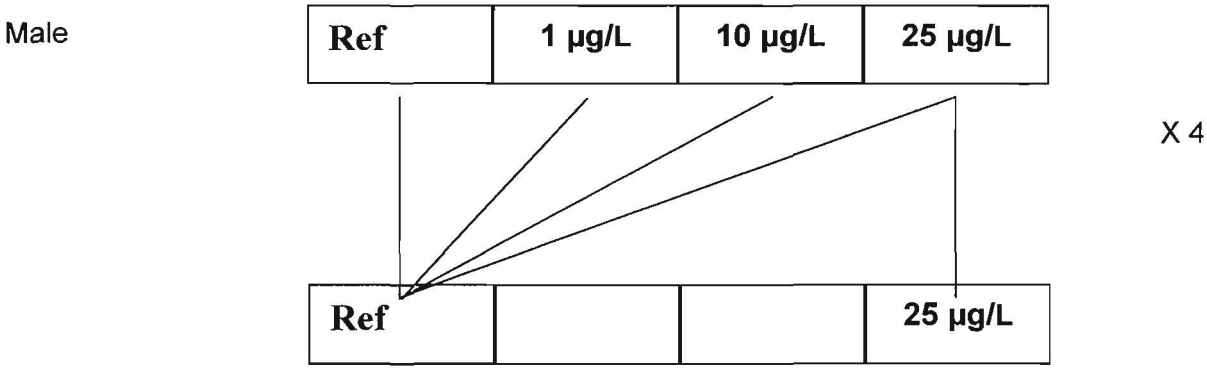


Figure 2.2. Schematic diagram of experimental design

To induce spawning, the male frogs were injected subcutaneously in the dorsal lymph sack with chorionic gonadotropin (pregnyl) for three consecutive days and the females were injected on days two and three only (Table 2.1).

Table 2.1: Amount of pregnyl injected in male and female *X. laevis* adult to induce spawning (Van Wyk *et al.*, 1984)

Day	Dose for	
	Male	Female
1	250 i.u	-
2	250 i.u	500 i.u
3	250 i.u	500 i.u

The day before injection was started, each frog's body (both male and female) was measured and the mass was taken.

Figure 2.3: A female *X. laevis*. Frogs were cryobranded according to the tank number. Note the number 3 on the abdomen.

After receiving the last injection, males and females were placed together as pairs into breeding tanks which were 300 x 240 x 240 mm in size. Each tank was fitted with a raised mesh floor to protect the eggs (fig. 2.3 & 2.4)

Figure 2.3: A breeding tank with a male and female

Figure 2.4: Frogs mating in the breeding tank

The day after spawning, the frogs were removed from the tank and the eggs were counted to determine the total number of eggs oviposited by individual frogs and the water containing embryos was aerated (fig. 2.5).

Figure 2.5: Tank containing eggs oviposited by a female frog

When the tadpoles were three days old they were counted. This was achieved by photographing the tadpoles digitally at high resolution in a 230 x 320 mm shallow white tray containing water to a depth of 2 cm (fig. 2.6). The photographs were imported into PowerPoint and overlaid with an 8 x 6 grid to assist towards counting the larvae (fig. 2.7). Fifty tadpoles were randomly siphoned from each breeding tank to glass jars using a silicone tube. The tadpoles were poured into 30L glass aquaria containing 25L constantly aerated fetax medium and were maintained there until they reached stage 66 (figs. 2.8, 2.9 & 2.10). Three duplicate tanks were set up for each breeding combination. Each breeding combination as well as each atrazine concentration was colour-coded. To prevent contamination, each atrazine concentration also had colour-coded dedicated nets, pipes and cleaning equipment (fig. 2.9).

Figure 2.6: Three day-old tadpoles in a shallow white tray

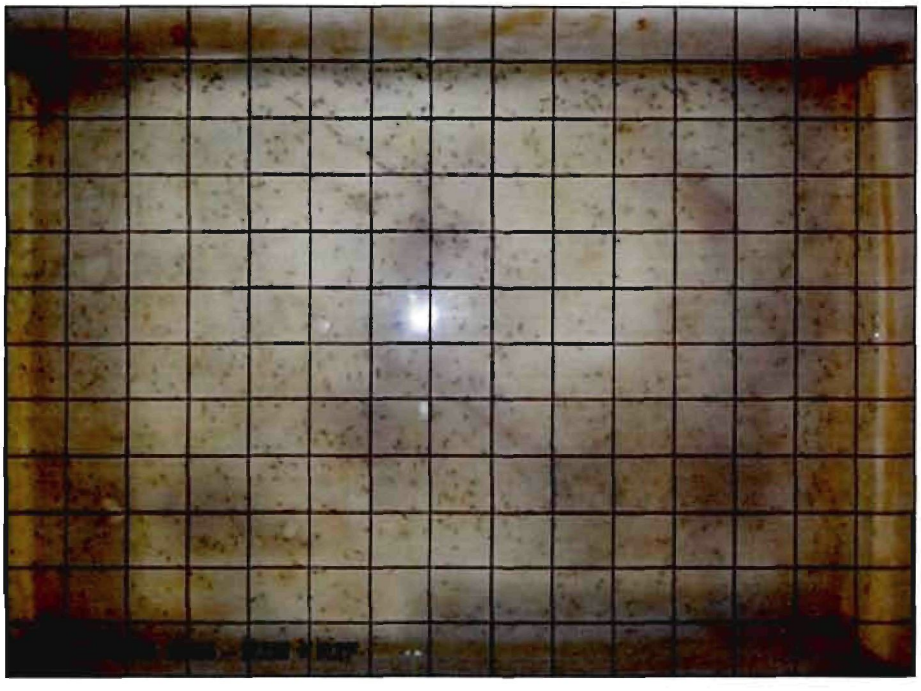


Figure 2.7: Photograph of larvae imported into PowerPoint and overlaid with a grid

Figure 2.8: Glass aquaria containing developing tadpoles

Figure 2.9: Tanks contain different concentrations of atrazine and glass aquaria with growing tadpoles set up in a temperature-controlled wet laboratory

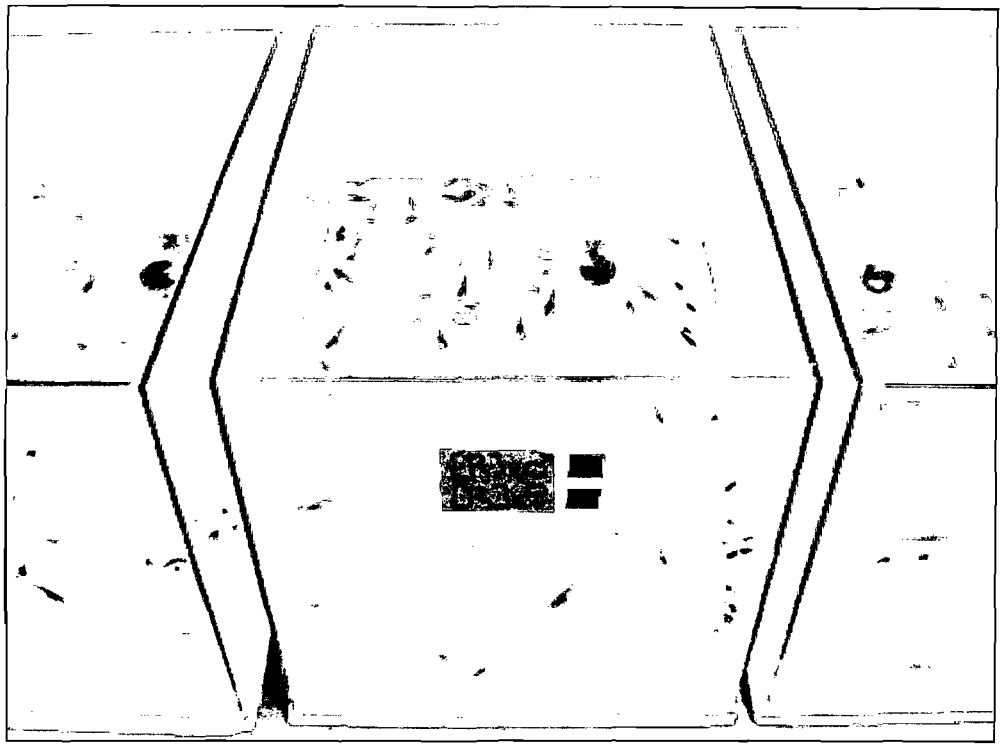


Figure 2.10: Glass aquaria with developing tadpoles

2.1.3 Feeding

Tadpoles were fed every second day with *Xenopus* pellets. Pellets were custom-manufactured by Avi Products and their manufacture was based on the formula of the company (Xenopus 1). 50 g of pellets were soaked in 100 ml tap water, liquidised with a food processor and then homogenised in 500 ml of tap water.

Table 2.2 Composition of *Xenopus* pellets

Protein	160g/kg
Moisture	120g/kg
Fat	25g/kg
Fibre	170g/kg
Calcium	18g/kg
Phosphorous	7g/kg

Tanks were cleaned once a week and filled with new Fetax medium containing the required atrazine concentration. Tadpoles were checked daily to identify developing metamorphs and counted weekly to determine survival.

2.1.4 Metamorphs

As tadpoles reached completion of metamorphosis (Niewkoop & Faber stage 66) they were removed from the tanks, anaesthetised using 0.1% solution of 3-amino benzoic acid ethyl ester (MS 222). Then the body mass was determined using an electronic Sartonus BP2105 scale (0.0001g accuracy) and Snout–vent length was measured to the nearest 0.1mm by means of a Teflon Vernier Caliper. After all the data was collected, a small cut was made on the abdomen to allow penetration of the fixture and a tag with an identification number was attached to the right hind leg of the frog. Specimens were fixed in Bouin's for 48 hrs, rinsed in water and then transferred to 70% ethanol. All the frogs were then dissected to expose the gonads for gross morphology. The sex of each frog was determined and gonads were digitally photographed using a Nikon Coolpix 900 digital camera fitted on a Nikon SMX 1500 dissecting microscope. Gonads were measured and examined externally (fig. 2.11). Gonads of all frogs were surgically removed by dissection and preserved in 70% ethanol.

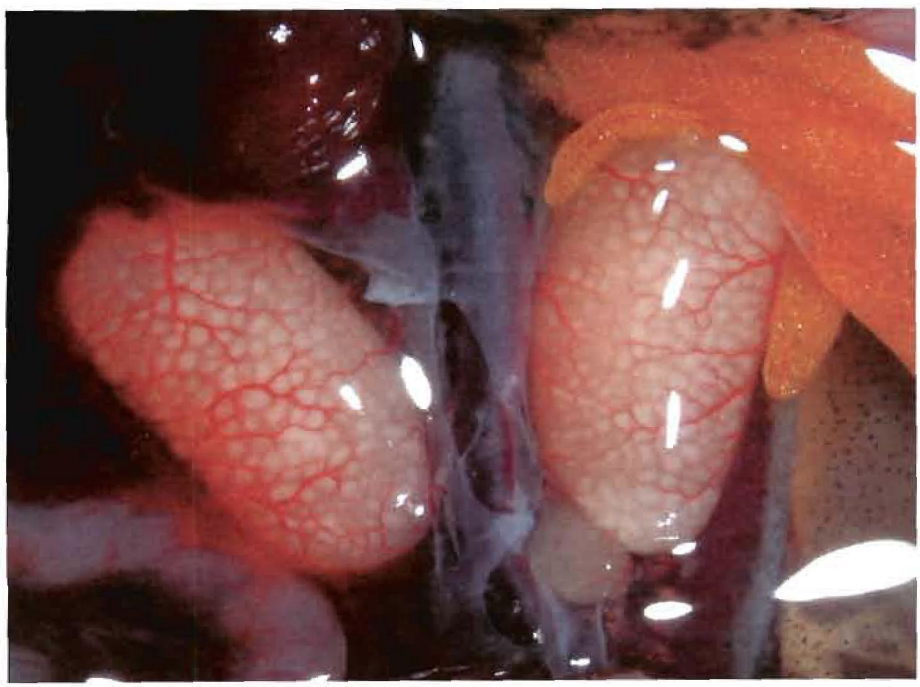


Figure 2.11. Testes with yellow fat bodies indicating a healthy animal.

2.1.4 Histology of metamorphs.

10 testes of the F_2 frogs were randomly selected from each breeding cross and prepared for histological examination. The preserved testes were dehydrated in graded alcohols embedded in paraffin wax and were then longitudinally serially sectioned at 6µm using a Reichert Jung 2050 microtome. Sections were stained with Harris haemotoxylin and eosin, then permanently mounted in DPX mounting medium. The prepared slides were examined using a Nikon Alphaphot compound microscope. All the sections were examined and the number of testicular ovarian follicles counted and recorded.

The mature F_1 frogs which were used in the cross-breeding experiments were anesthetised with 0.1% solution of 3-amino benzoic and ethyl ester (MS222), weighed, snout-vent length was measured, and they were examined externally. They were dissected and the gonads were exposed and photographed with a Nikon Coolpix digital camera attached to a Nikon SMZ1500 dissecting microscope. The gonads were removed, measured and weighed. One testis of each male was fixed in Bouins solutions for 48 hours and then transferred to 70% ethanol and prepared for sectioning and histological examination. The ovaries and the other testes of each male were stored in a freezer.

2.2 Testicular ovarian follicle prevalence in *X. laevis* from atrazine-free localities in South Africa.

2.2.1 Source of frogs

Male frogs were collected from seven areas which were situated in two different geographical areas. Four areas were in the northern part of the Cape and three areas were in the western part of the Cape (fig. 2.12). Site A was in the central highveld (Potchefstroom), site B in the northern Karoo (Sophiasdal, Reddersburg), site C in the greater Karoo (Koka Tsjara, Beaufort West) and site D in the Little Karoo (Jacques Well, Laingsburg) - all these areas are in the northern part of the Cape. From the Western Cape, frogs were collected from site E (Jonkershoek, Stellenbosch), site F (Jonkershoek Hatchery, Stellenbosch) and site G (Klapmuts, Bellville).

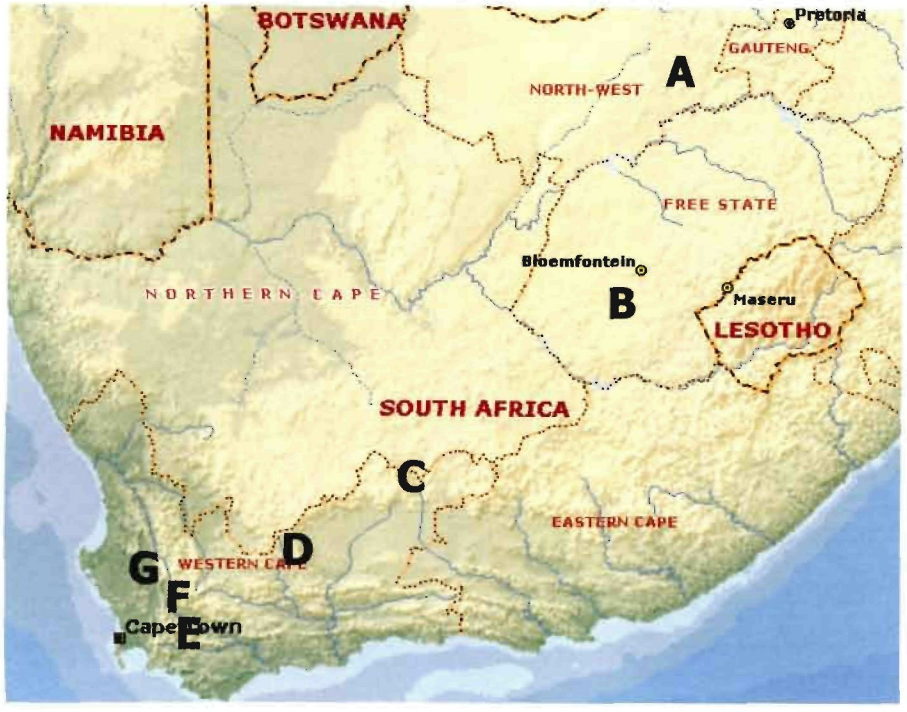


Figure 2.12. Sampling sites

2.2.2 Evaluation of sites

Each marked site was evaluated and characterised to determine if it would be suitable for this study. At each site the catchment area was inspected to determine what the land was used for, and water and sediment samples were collected.

2.2.3 Collecting and processing of specimens

Four to ten baited bucket *Xenopus* traps were set in the selected water bodies. Traps were baited with beef liver in a gauze bag to prevent the frogs from swallowing the bait. A sediment grab sample was taken in shallow water at the four wind directions around the pond. Four 1L water samples were collected, one from each quadrant of the water body (SOP for water sampling). These samples were pooled and two 1L sub-samples collected in 1L solvent-rinsed (acetone and hexane) glass bottles (SOP for water analysis). Water samples were stored at 4°C (not frozen) (Eisenreich *et al.*, 1994) for the analysis of atrazine and terbuthylazine and chloro-metabolites in environmental samples, as well as other pesticides. Analyses for triazines were conducted by Dr Robert Yokley (Syngenta Laboratories) and analyses of other pesticides and elements in sediment

and water by the South African Bureau of Standards, a certified laboratory located in Pretoria, South Africa. Water quality parameters were recorded.

The thorax of the anaesthetised frog (MS 222) was opened and a blood sample collected directly from the ventricle with an EDTA-rinsed insulin syringe and needle. Blood samples were transferred to EDTA-rinsed Eppendorf vials and kept on ice before centrifuging (Eppendorf Centrifuge 5804R) at 10 000 rpm for three minutes. The supernatant was transferred to a labelled cryo vial and the Eppendorf vials with cell components stored at -80°C. All specimens were closely inspected for malformations and other abnormal morphological characteristics. The snout-vent lengths of the frogs were measured by means of a Vernier Calliper (±0.1 mm). Frogs were weighed in an empty 600-ml plastic bottle on a Sartorius BP210S balance (± 0.01 g). After gross morphological inspection, frogs were dissected and the gonads measured and photographed. Gonads were examined for testicular anomalies and photographed. One gonad was then flash-frozen in liquid nitrogen and stored at -80°C for hormonal and enzymatic analysis. The second gonad was placed in a biopsy cassette and fixed in Bouin's fixative for 48 hours for histological examination. Biopsy cassettes, with tissue, were then transferred to 70% ethanol for storage. The age of specimens was determined through skeletochronology by collecting the longest toe of one hind leg and fixing it in Bouin's fixative then preserving it in 70% ethanol. All carcasses were labelled and frozen.

2.2.4 Histological examination

The preserved testicular tissues were dehydrated in graded alcohols embedded in paraffin wax and then longitudinally sectioned at 6 µm using a Reichert Jung 2050 microtome. Sections were stained with Harris haemotoxylin and eosin, then permanently mounted in DPX mounting medium. The prepared slides were examined using a Nikon Alphaphot compound microscope. The number of testicular ovarian follicles were counted and recorded.

2.2.5 Skeletochronology

To determine the age of dissected animals, the second last and last digit from one of the toes were removed, fixed in Bouin's, decalcified in Perenyii solution, sectioned and stained with Erlich haematoxylin and eosin. Slides were interpreted and the age of frogs was determined according to the African Amphibian Conservation Research Group SOP for Skeletochronology (LDP-05A).

Chapter 3

Results

CHAPTER 3

Results

3.1 Cross-breeding

3.1.1 Number of eggs laid

All breeding combinations were successful and produced viable egg clutches. The average number of eggs produced, however, varied between breeding combinations (fig. 3.1.). For the Reference-Reference (R-R) combination an average 4 165 eggs were produced with a maximum of 6 717. The 1-R combination produced an average of 1 486 with a maximum of 1 905, the 10-R combination produced on average1 010 eggs with a maximum of 1 606, the 25-R combination produced on average 1 586 with a maximum of 1 960, and the 25-25 combination produced an average of 2 169 with a maximum of 4 192 eggs. The number of eggs laid was corrected for the mass of the females (fig. 3.1).

Even though the frogs were randomly selected, it so happened that some of the female frogs used in the R-R breeding combination were significantly larger than those in other concentrations. On average, the females in R-R weighed 49,3 g, compared to 54,9g in 1-R, 32,5g in 10-R and they were at 34,3g in the 25-25 combination.

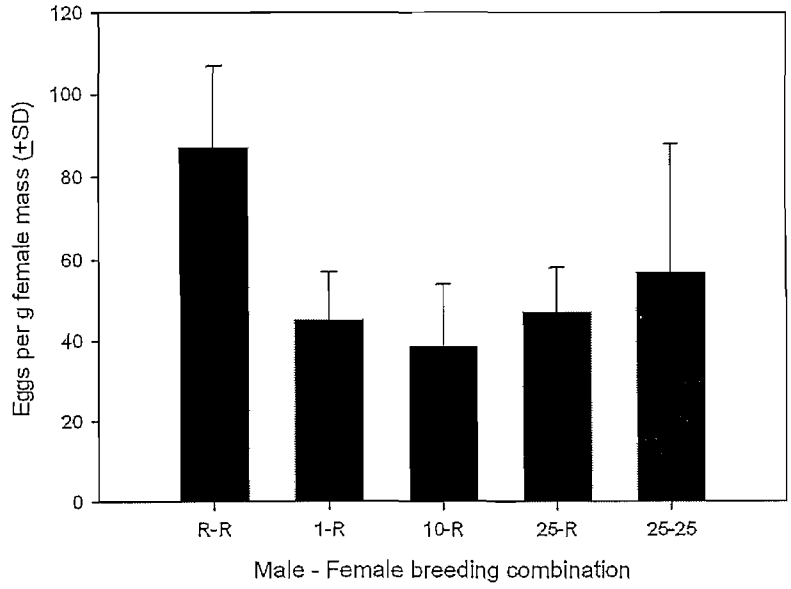


Figure 3.1: Weight-corrected number of eggs laid per F1 female

3.1.2 Percentage of eggs hatched

Eggs developed in all breeding combinations and no significant differences were observed between different combinations (fig. 3.2.). The percentage of eggs that hatched varied from 93% for the 25-R combination to 58.3% for the 10-R combination. The 1-R combination had a hatching success of 83%, the R-R combination a success rate of 74.8% and the 25-25 combination, 63.3%.

The mean sizes of the male frogs in the tanks were 31,6g in R-R, 23,4g in 1-R, 26,7g in 10-R, 19,6g in 25-R and 17,79g in 25-25. It was interesting to note that, although R-R had the largest males, they did not produce the greatest number of eggs nor had the highest hatching success. The combination of 25-R had an average of 19.6g males yet it had the highest number of eggs that hatched.

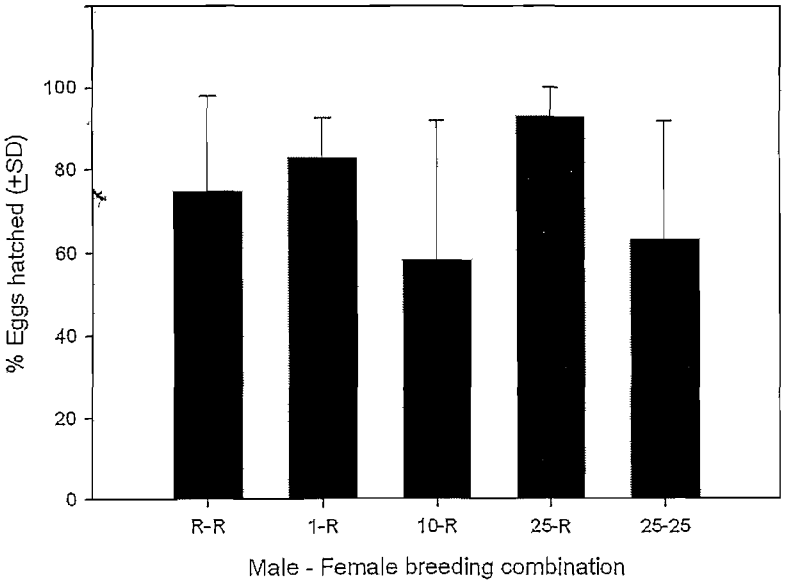


Figure 3.2: Percentage of eggs hatched at different breeding combinations

3.1.3 Days to first stage 66 metamorph

The number of days it took for all combinations to reach the first stage 66 was noted for all breeding combinations (fig. 3.3.) In all breeding combinations, tadpole development was satisfactory and the time it took for first tadpoles to complete metamorphosis did not differ significantly between any of the breeding combinations. Tadpoles in the R-R combination took on average 59 days to the first stage 66, compared to 60 days for 1-R; 63 days for 10-R; 65 days for 25-R and 63 days for 25-25.

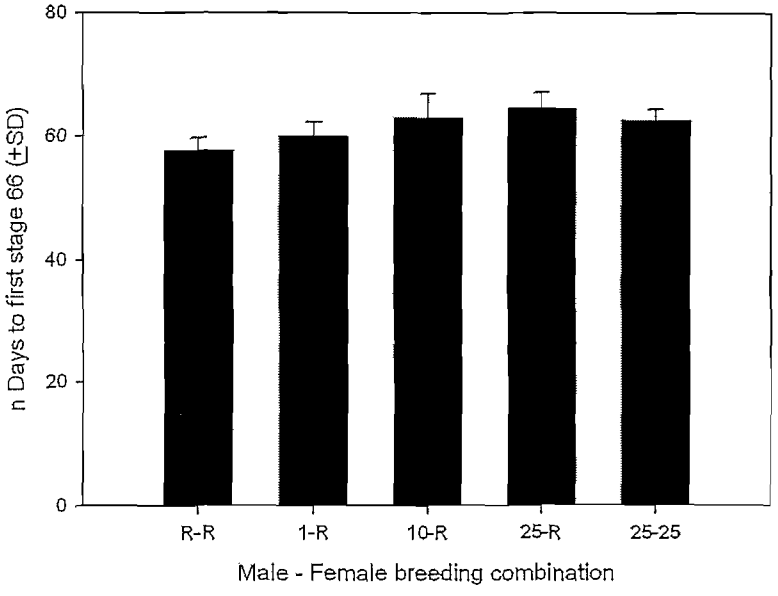


Figure 3.3: Number of days to first stage 66 of first F2 larvae

3.1.4 Days to last stage 66 metamorph

Although in some combinations a few tadpoles developed slower and took longer to complete metamorphosis (fig. 3.4.), the bulk of the specimens completed their metamorphoses in the same time and none of the combinations showed a delayed development. On average the number of days it took for the last tadpoles in each breeding combination to reach stage 66 was 99 days in R-R; 103 in 1-R; 98 in 10-R; 109 days in 25-R and 104 days in 25-25.

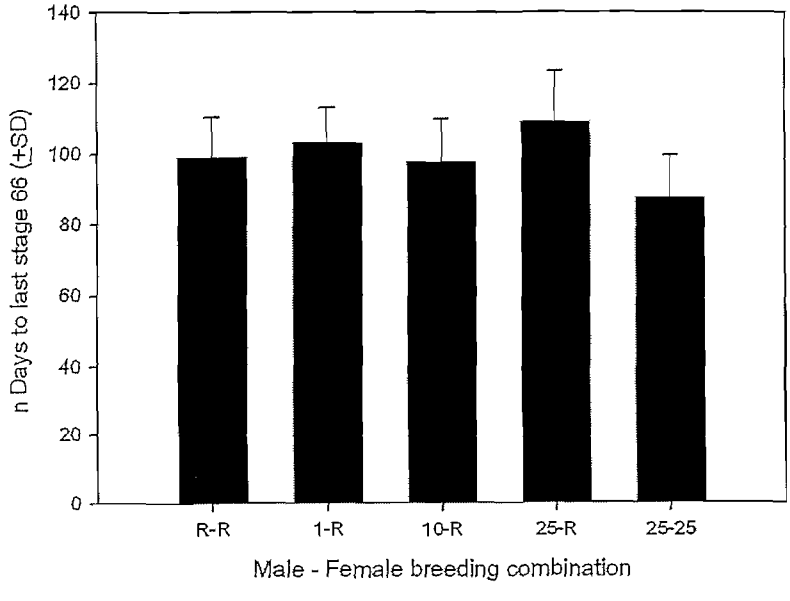


Figure 3.4: Number of days to the last stage 66 of last F2 tadpole

3.1.5 Percentage survival

The percentage of survival was recorded for different breeding combinations (fig. 3.5). The recorded survival was 84 % for the R-R combination; 87 % for 1-R; 82% for10-R;85% for 25-R 70 % for 25-25 No correlation was observed between atrazine concentration and tadpole survival.

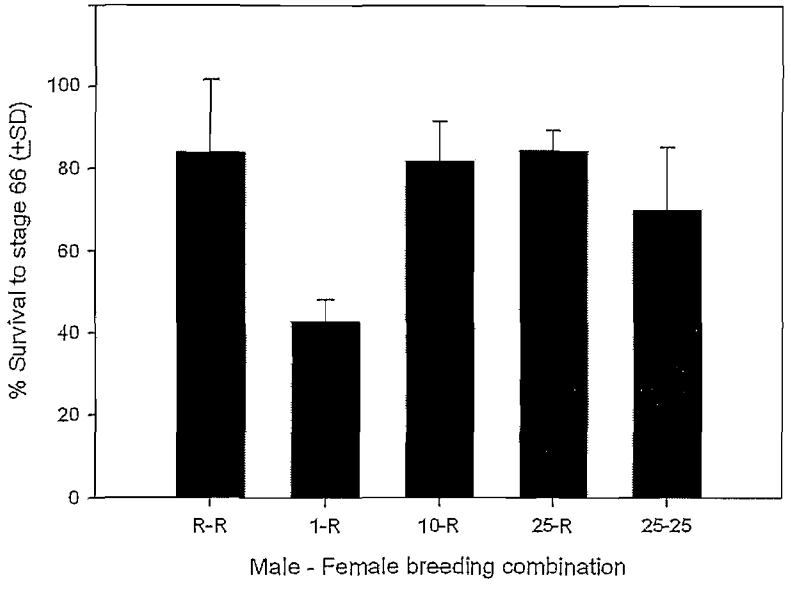


Figure 3.5: Percentage survival metamorphs in the breeding combination

3.1.6 Sex ratio

For all combinations, except the 25-R combination, slightly more females than males were produced (fig. 3.6). The percentage ratios of males to females was 45:55 for R-R combination; 43:57 for 1-R combination; 45:55 for10-R combination; 54:46 for 25-R combination; and 47:53 for 25-25 combination.

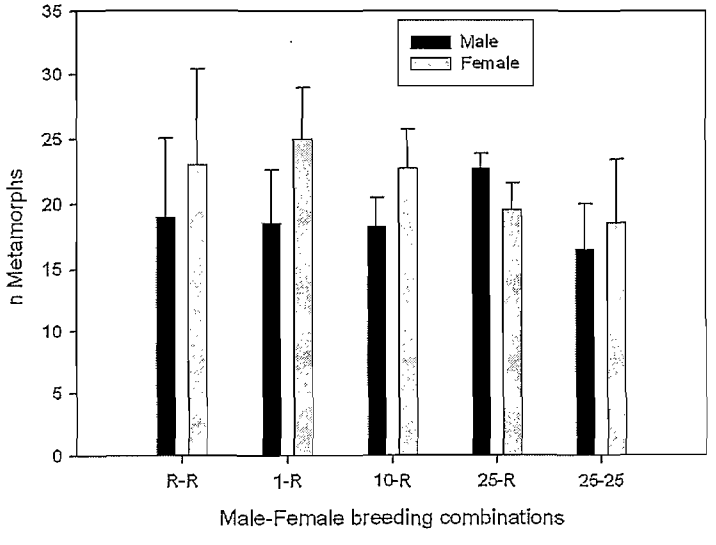


Figure 3.6: Sex ratio in metamorphs that survived

The snout-vent lengths (SVL) and masses of all F2 generation (both male and female) were measured and recorded. Analysis of variance (AVOVA) of the median values for the mass and snout-vent length for each of the breeding combinations produced p-values of 0.395 for mass and 0.166 for SVL, which means that there were no significant differences between the mass or SVL of the F2 frogs, but there appeared to be a concentration-related decrease in the mass of males that were exposed to 1,10 and 25µg/L atrazine. The mass and SVL of F2 frogs produced from pairings where both the parent male and female frogs had been exposed to 25µg/L were the same as those from the reference pairings. Linear regression (r^2) of the exposure concentration for the pairs was 0.107 for the median mass and it was 0.16 for the SVL of the frogs, showing no significant trend.

3.1.7 Gross anomalies and testicular ovarian follicles of crossbred generations

A subset of testes of the F2 frogs were serially sectioned and checked for morphological anomalies. Anomalies discovered included the complete absence of one testis and the presence of a discontinuous testis on one side (Table 3.1).

Table 3.1. Gross gonadal anomalies observed in the F2 generation metamorphs

Testes combination	% discontinuous testes	% one testis
R-R	2.6%	2.6%
1-R	1.4%	1.4%
10-R	1%	1%
25-R	0%	0%
R-R	0%	0%

Testicular ovarian follicles were observed in all breeding combinations (fig. 3.7). The observed prevalence of testicular ovarian follicles was 8% for the R-R breeding combination; 20% for 1-R; 7% for 10-R; 19 for 25-R; and 6% for 25-25. We thus did not find any dose-dependant effect. The mean number of ovarian follicles per affected animal was found to be 6 in R-R; 3 in 1-R; 18 in 10-R; 4 in 25-R;and 8 in the 25-25 breeding combination (fig. 3.8). Although the number of ovarian follicles varied significantly between treatments, no dose-dependant pattern was observed.

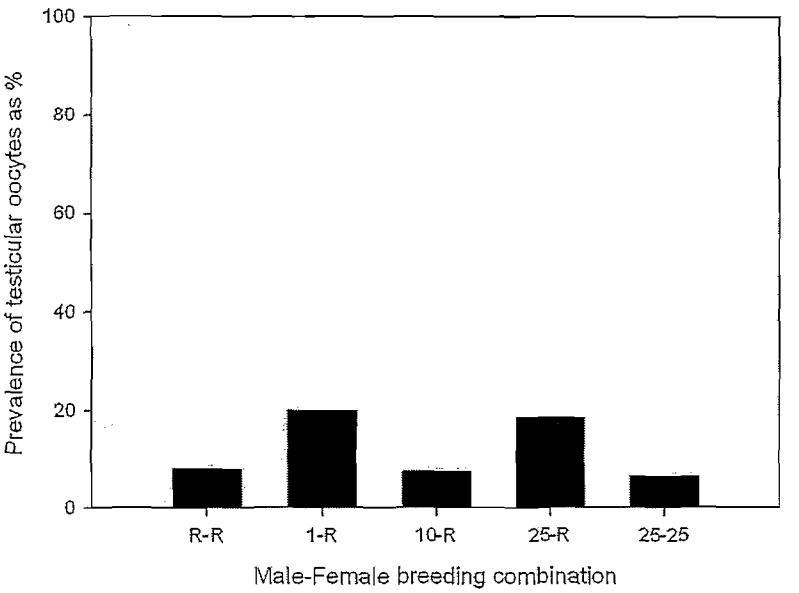


Figure 3.7: Prevalence of F1 generation with testicular ovarian follicles

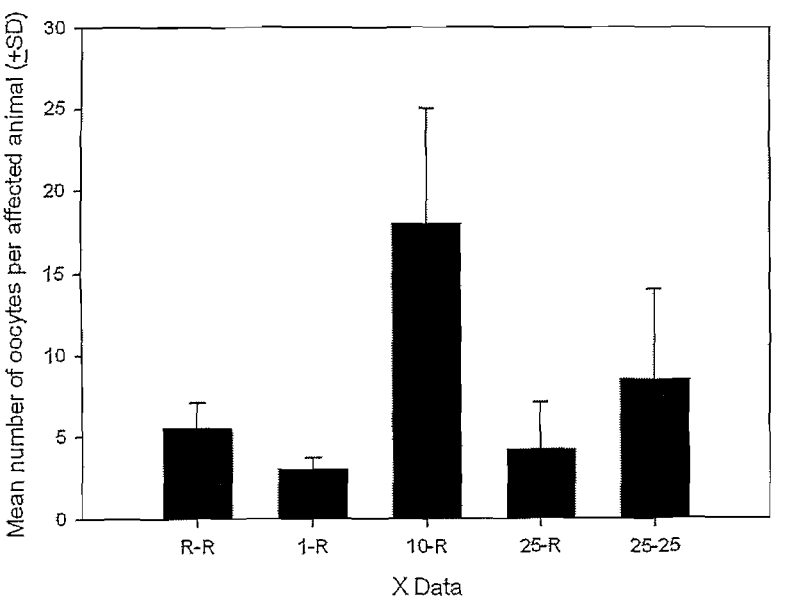


Figure 3.8: Mean number of testicular ovarian follicle per affected animal over the study period

3.1.8 Testicular anomalies observed in the breeding adults

Testes of all the frogs that were used in the breeding experiments were also serially sectioned and screened for testicular ovarian follicles. Jooste reported a decrease in the number of testicular ovarian follicles from stage 66 metamorphs through 10 month grow-out. We observed a continuation of this reduction at 30 months' grow-out. After 30 months there were only regressed

ovarian follicles in the different concentrations except in the 25 µg/L instance, where both mature and regressed ovarian follicles were observed (figs. 3.9 & 3.10.).

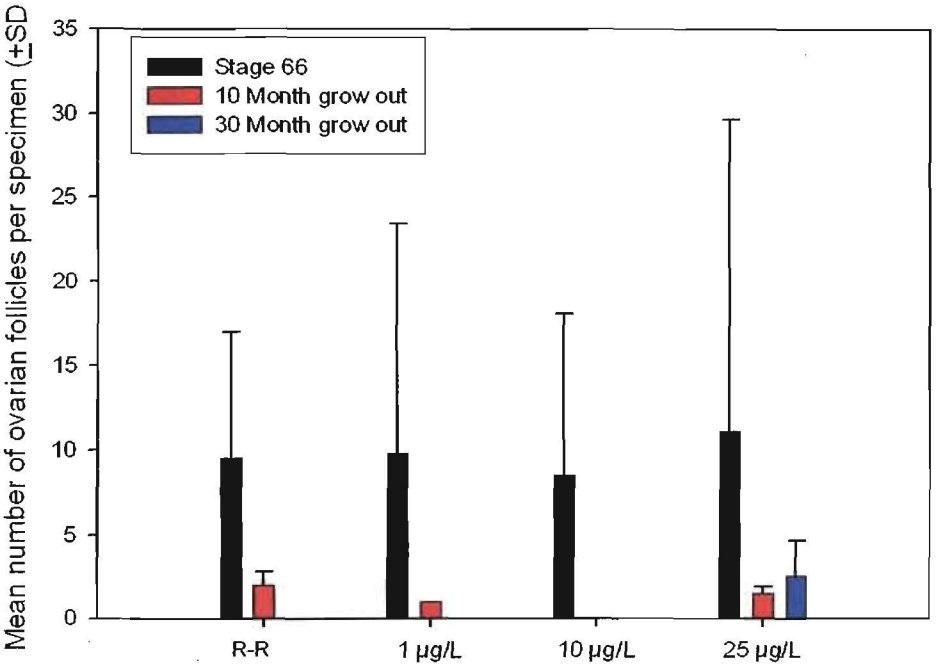


Figure 3.9: Mean number of testicular ovarian follicles per specimen in the atrazine concentrations

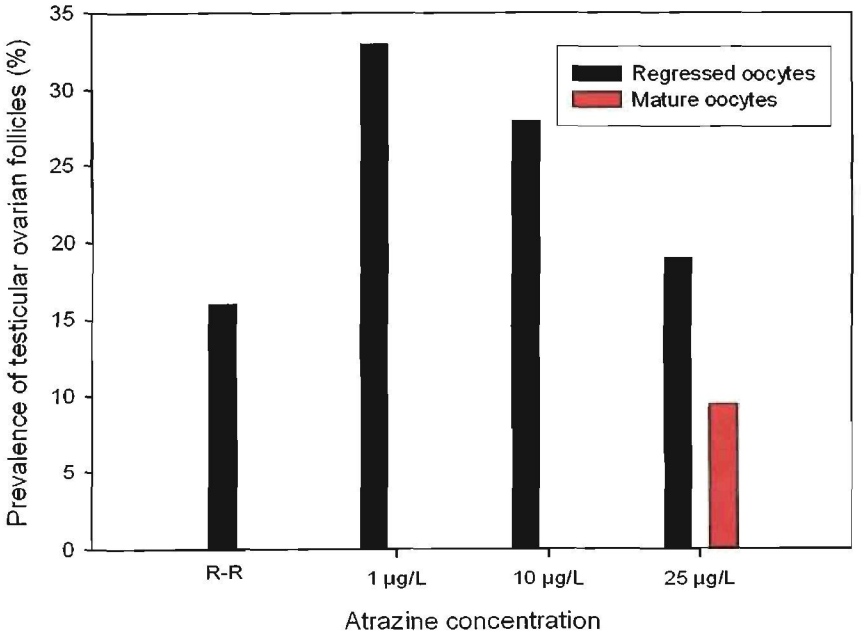


Figure 3.10: Prevalence of regressed and mature ovarian follicles after 30 months in the respective atrazine *concentrations*

3.2. Prevalence of testicular ovarian follicles under natural conditions

3.2.1 Physical properties and land use of various sites

The physical properties and land use were determined for all the sites as pointed out in figure 2.X.

Table 3.2. Site A Taggart Farm, Potchefstroom

Grid reference	26.594444S 27.196388"E
Surface area (Sept 2001)	20,500 m^2
Watershed area	244 ha
Source of water	Rainfall + fountain feeding into dam
pH	8.3
Dissolved oxygen	3.39 mg/l
Surrounding vegetation	Wooded thornveld. Natural vegetation. No crops in catchment. Cattle farm.
Land use	This site is being used a water point for cattle. No agrochemicals were applied in the catchment area and cattle do not receive any growth hormone injections.

Table 3.3. Site B Sophiasdal, Reddersburg

Grid reference	29.45234S 26.19058E
Site description	Earth-walled farm pond
Surface area (Sept 2001)	60 000 m^2
Watershed area	50 ha
Source of water	Rainfall
pH	8.4
Dissolved oxygen	76%
Surrounding vegetation	Open grass veld and Karoo shrubs
Land use	Watering point for sheep

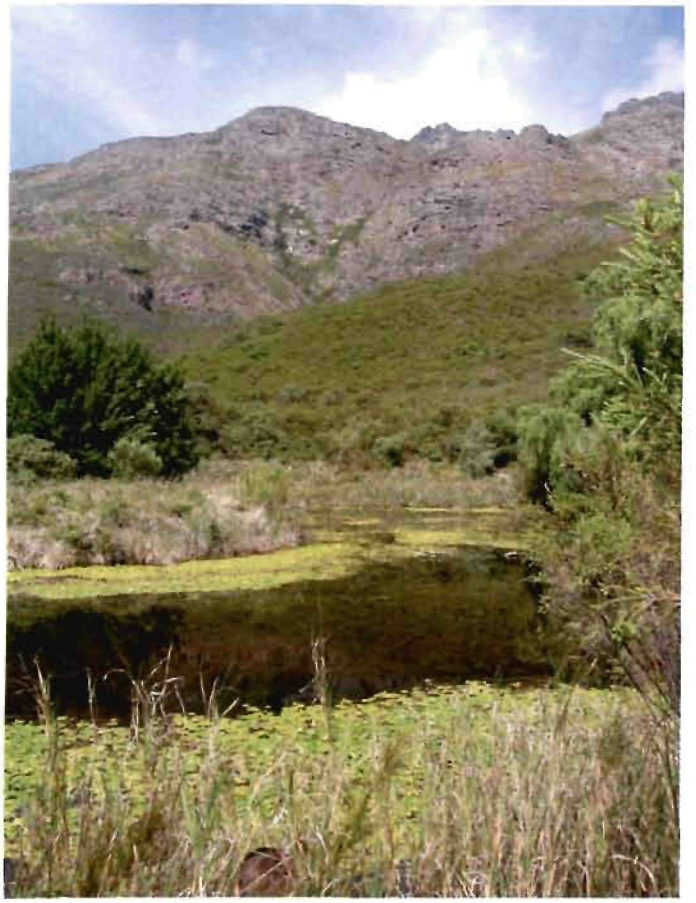
Table 3.4. Site C Ko ka Tsjara, Beaufort West

Grid reference	32.241388S 22.583611E
Site description	Small pond below the dam wall of a very large dam
Surface area (Sept 2001)	250 m^2
Watershed area	Below overflow of large dam with very large catchment
Source of water	Rainfall + dam
pH	7.3
Dissolved oxygen	6.2mg/l
Surrounding vegetation	Karoo shrubs, Phragmites, Acacia trees
Land use	This dam has a very large catchment area and serves as reservoir for the town of Beaufort West. The catchment area of this site is predominantly sheep farming.

Table 3.5. Site D Jacques Well, Laingsburg

Grid reference	33.27505S 20.84781"E
Site description	This site is a natural spring and receives no run-off as it is surrounded by an earth wall
Surface area (Sept 2001)	50 m^2
Watershed area	None. No run-off water enters this site.
Source of water	Fountain
pH	7.8
Dissolved oxygen	5.12mg/l
Surrounding vegetation	Dense stand of *Phragmites*
Land use	Water is used for irrigation of close-by olive and apricot orchards as well as onion fields. Herbicide "Goal" with active ingredient oxyfluor and "Gallant" with active ingredient haloxyflor-R methyl ester were used on crops.

Table 3.6. Site E Jonkershoek, Stellenbosch



Grid reference	33.963027S 18.926305E
Site description	Earth-walled pond at the foot of the Jonkershoek mountain
Surface area (Sept 2001)	300 m^2
Watershed area	10 ha mountain slope
Source of water	Rainfall
pH	4.94
Dissolved oxygen	0.53 mg/l
Surrounding vegetation	Cape fynbos
Land use	Part of nature reserve.

Table 3.7. Site F Jonkershoek Hatchery, Stellenbosch

Grid reference	33.966666S 18.950000E
Site description	Earth-walled pond
Surface area (Sept 2001)	1000 m^2
Watershed area	20 ha
Source of water	Rainfall against mountain slope
pH	7.10
Dissolved oxygen	5.6 mg/l
Surrounding vegetation	Lawns and willow trees
Land use	Home of the Stellenbosch Trout Angling Club. This site forms part of a series of six ponds.

Table 3.8. Site G Klapmuts, Bellville_

Grid reference	33.816666S 18.866666 E
Site description	Earth-walled dam in a valley covered with vineyards. This site receives run-off from the vineyards
Surface area (Sept 2001)	10 000m^2
Watershed area	100 + ha
Source of water	Rainfall
pH	6.9
Dissolved oxygen	mg/l 53.5%
Surrounding vegetation	Surrounded by vineyards
Land use	Irrigation pond for vineyards

3.2.2 Chemical analyses of water samples

Table 3.9 shows that there was no detection of organochlorines, organophosphorous, pyrethroids, PCBs and Triazines in all the locations selected - except in site G where a low concentration of triazines was detected. This could be expected since this site collected water from agricultural land.

Table 3.9: Chemical compounds detected in water samples. (ND – not detected). Detection limit 0.01µg/L.

Site	Organochlorines in µg/ℓ	Organophosphorus in µg/ L	Pyrethroids in µg/L	PCB's in µg/L	Triazines in µg/ℓ
A	ND	ND	ND	ND	ND
B	ND	ND	ND	ND	ND
C	ND	ND	ND	ND	ND
D	ND	ND	ND	ND	ND
E	ND	ND	ND	ND	ND
F	ND	ND	ND	ND	ND
G	ND	ND	ND	ND	Atrazine: 0.1 Simazine: 0.4 Terbuthylazine: 0.3

3.2.3 Frogs collected

The frogs were collected at different dates as indicated in the table below. In some sites we had difficulty to collect the target of 50 males (Table 3.10).

Table 3.10. Numbers of frogs collected at selected sites

Site	Date	N Males	N females
A	6 Sept 2003	50	122
B	20 June 2006	60	198
C	16 Nov 2005 2 May 2006	12 0	18 0
D	15 Nov 2005	>100	>100
E	14 Nov 2005 4 May 2006	1 14	4 15
F	5 May 2006	18	25
G	4 May 2006	27	80

Frogs collected north of the Cape Fold Mountains (sites A-D) had blotches (fig. 3.11A.) while those collected south of the Cape Fold Mountains (sites E-G) were more mottled and were characterised by the striking appearance thereof (fig. 3.11B).

A. B

Figure 3.11: Clawed frogs collected during the present study. A (Potchefstroom, Site A) and B (Klapmuts, Site G)

3.2.4 Body mass and snout-vent length

The average snout-vent lengths of frogs from all sites were not significantly different and ranged between 59mm and 70mm (fig. 3.12.). The largest frogs were collected at Jonkershoek (site E) while the smallest were collected in the Karoo at Reddersburg (site B). A significant variation in the body mass of the frogs was observed. Generally those collected from the Western Cape area (sites E-G) were heavier than those collected from the northern area (sites A-D) (fig. 3.13.). As can be expected, the heaviest frogs were collected at Jonkershoek (site E) while the lightest were collected in the Karoo at Reddersburg (site B).

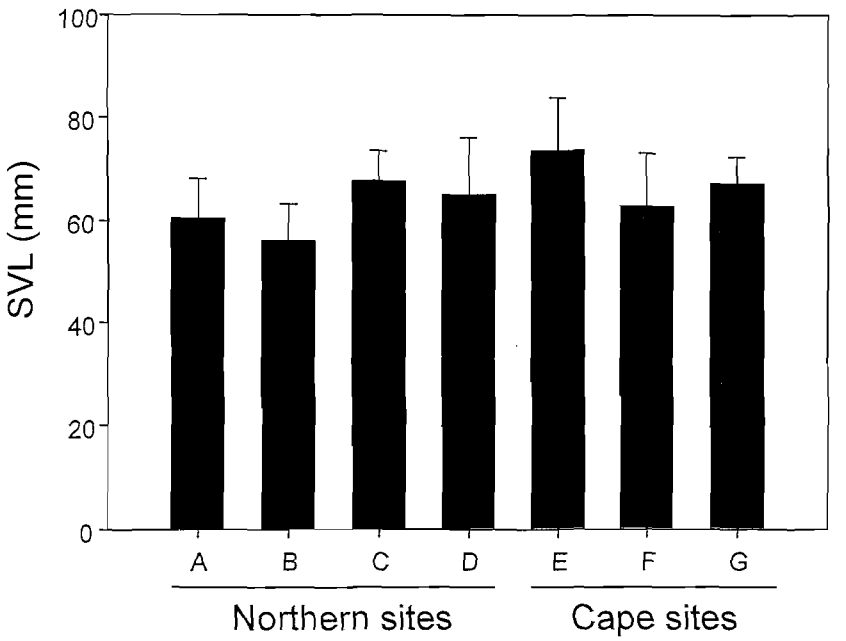


Figure 3.12: Snout-vent length (SVL) of male frogs collected plus standard deviation(SD)

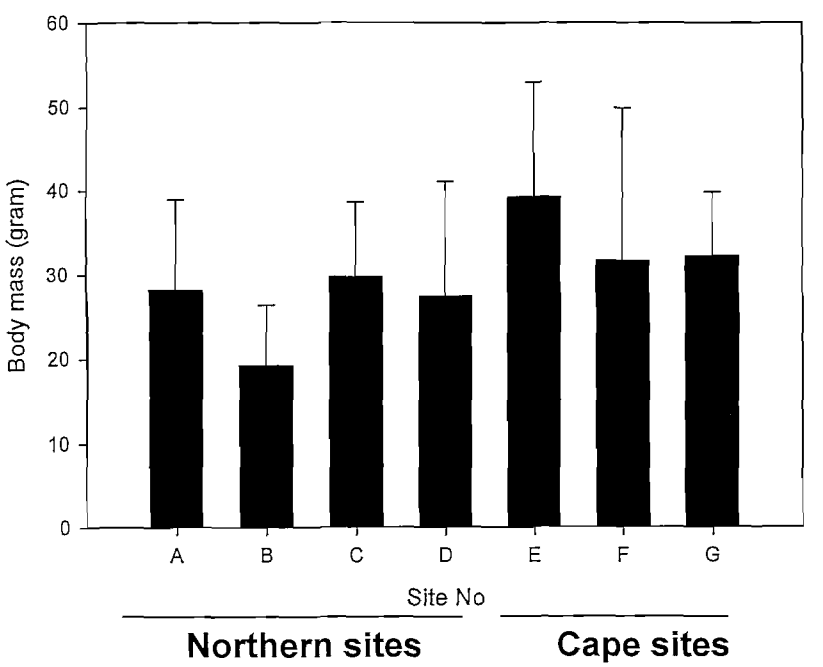


Figure 3.13: Body mass of male frogs collected plus standard deviation

3.2.5 Gonad measurement

The weights of the testes from the different sites differed significantly and mean gonado-somatic indexes were calculated for all sites (fig. 3.14.). We observed significant variations between sites, but a close correlation between body size and gonad size. The largest gonads were collected in frogs from Jonkershoek (site E) while the smallest were from frogs collected in the Karoo at

Reddersburg (site B). The length of the testes varied from 5mm to 9.5mm within the different sites (fig.3.15).

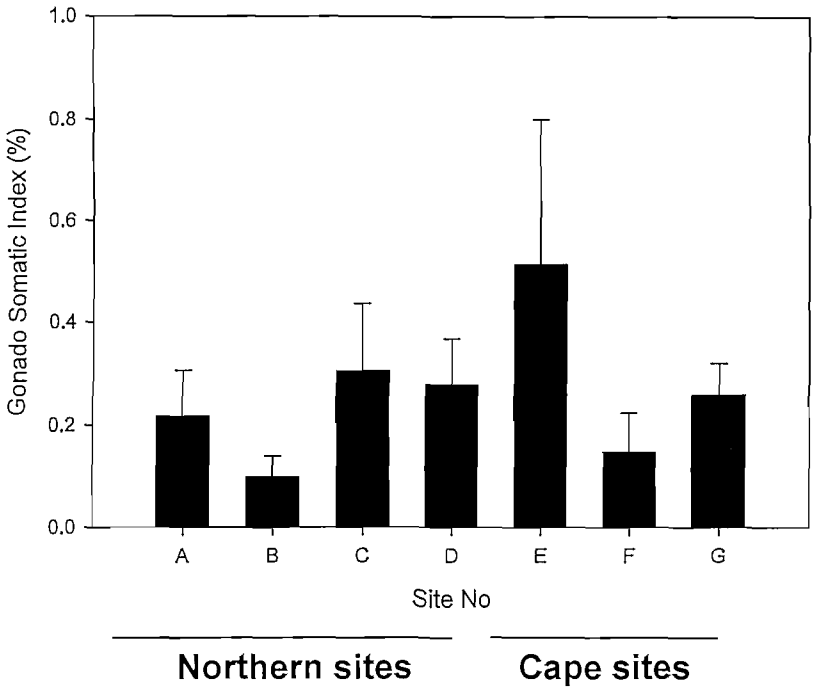


Figure 3.14: Gonado-somatic indexes for male *Xenopus laevis*

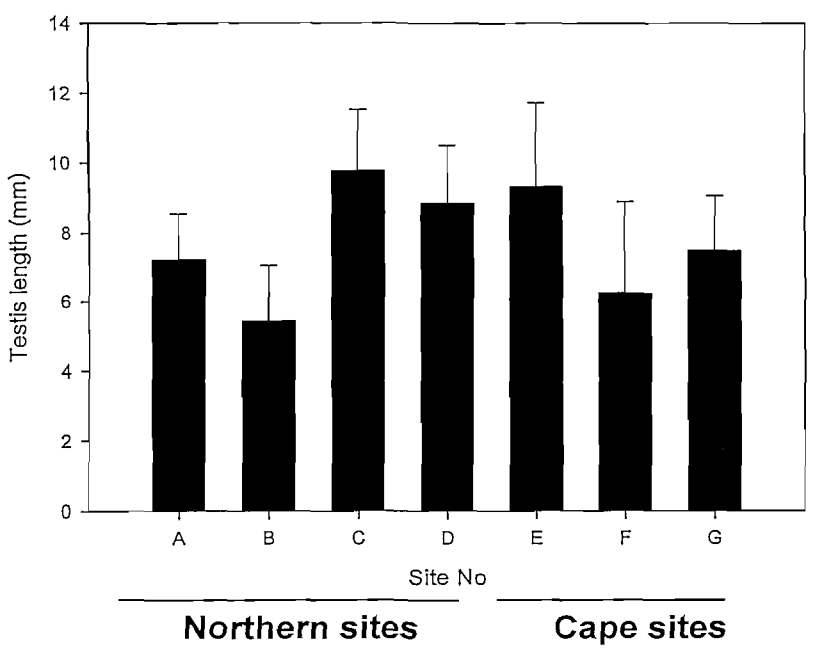


Figure 3.15: Length of testes *Xenopus laevis*

3.2.6 Testicular ovarian follicles

Testicular ovarian follicles were observed in the testes of frogs collected from sites A to D while none of the frogs from the Cape sites had any. In males from sites A and B both regressed and mature ovarian follicles were observed. (fig. 3.16 A & B). Frogs from site C only had mature ovarian follicles and those from site D had only regressed ovarian follicles.

The average number of ovarian follicles per individual was also significantly low for all sites - except site A, the frogs of which had an average of 55 regressed ovarian follicles (figs. 3.17 & 3.18).

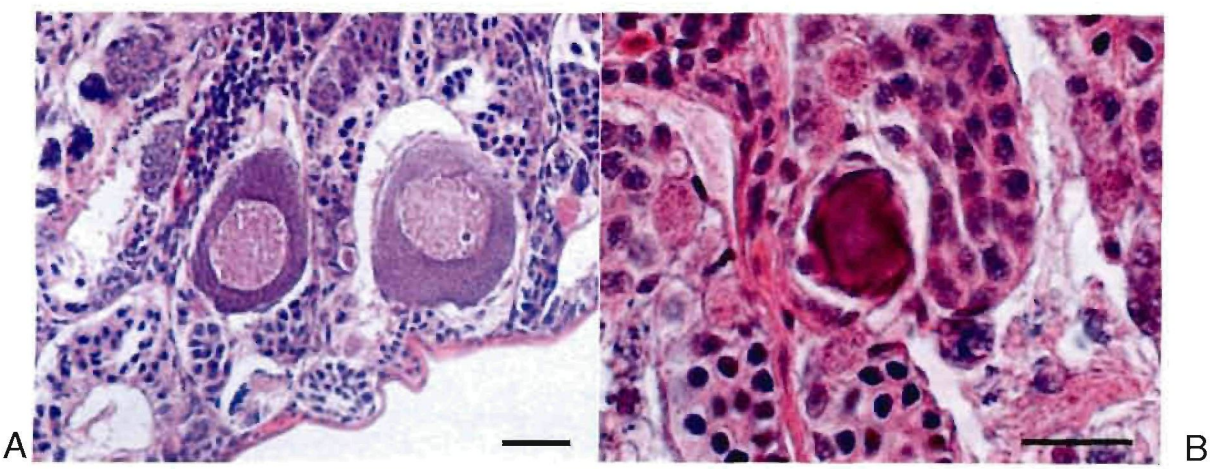


Figure 3.16: Testicular ovarian follicles. A – Mature follicle, scale bar = 100μm; B – Regressed follicle, scale bar = 30μm

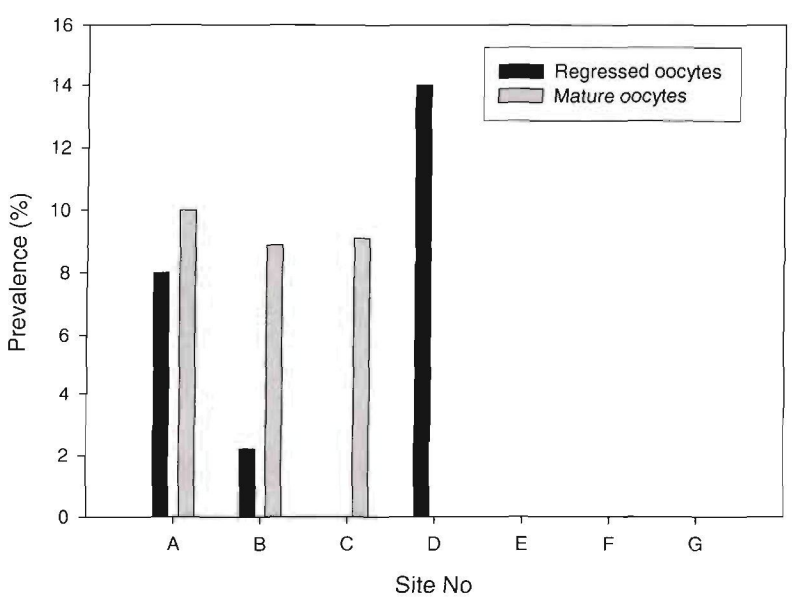


Figure 3.17: Prevalence of testicular ovarian follicles observed

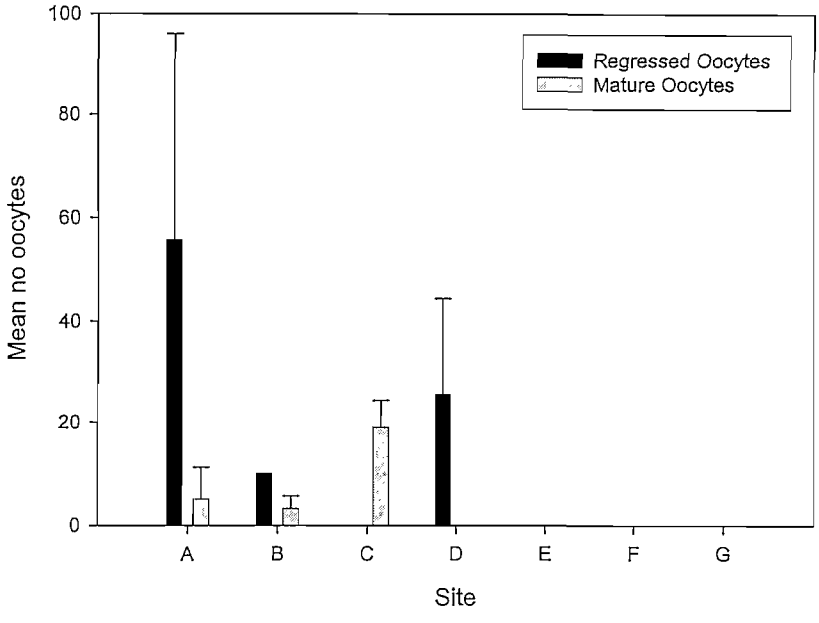


Figure 3.18: Mean number of testicular ovarian follicles per individual

3.2.7 Age profile

Ages of the collected frogs ranged from one year to six years with a mean of around two years (fig. 3.19). The age profile also varied for the different sites with sites B, E, F and G yielding a large number of frogs that were one year old; from site A, B and D a large number of two year-olds were collected and site D had a large number of three year-olds. Only site D had specimens (two) that were six years of age. (Table 3.11; figs. 3.19 & 3.20).

Table 3.11: Summary of ages profiles for frogs collected

	1 Year	2 Years	3 Years	4 Years	5 Years	6 Years
Site A	14	20	12	3	1	0
Site B	30	14	1	0	0	0
Site C	3	4	4	1	0	0
Site D	6	14	15	9	4	2
Site E	16	6	4	2	2	0
Site F	10	1	3	1	1	0
Site G	17	1	2	1	2	0

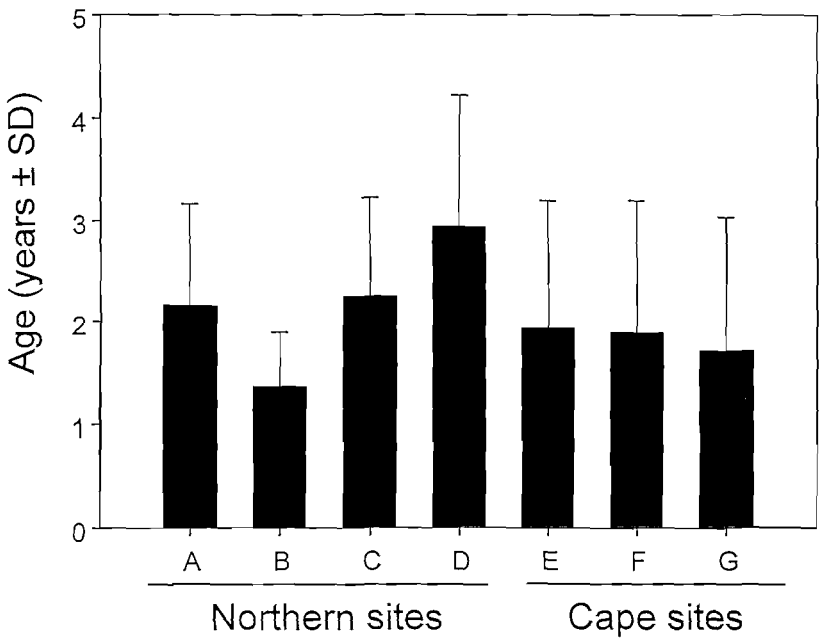


Figure 3.19: Mean age of male frogs at collecting sites

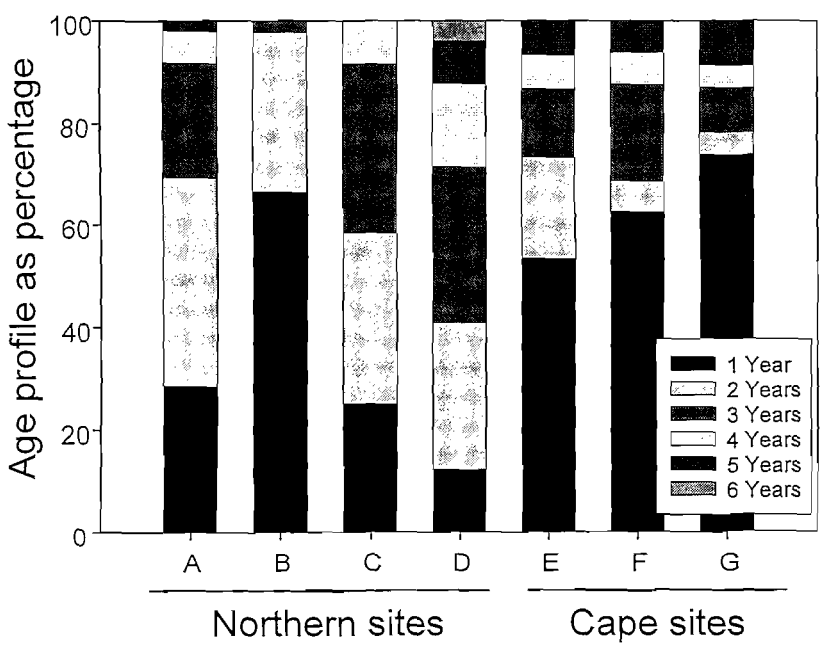


Figure 3.20: Age profile for *Xenopus* collected at various sites

Chapter 4

Discussion

CHAPTER 4

Discussion

4.1 Cross-breeding

Continuous exposure of *X. laevis* from egg through to 24 months of age to concentrations of 1, 10 and 25 µg/L showed no detectable adverse effects on the F1 generation or on the development and survival of the F2 generation. Jooste *et al.* (2005) reported that no concentration response was detected through to completion of metamorphosis, and his animals were also used for the cross-breeding experiments conducted for purposes of the present study. In this study we found that there was no correlation between atrazine exposures and hatchability, time to metamorphosis or body size at metamorphosis. This suggests that the developmental capacity of offspring of atrazine exposed frogs is not compromised (Wilbur & Collins, 1973) by atrazine exposure. In spite of the fact that atrazine has been in use for more than four decades, robust populations of clawed frogs still exist in areas of intense crop production and atrazine use (Hayes *et al.*, 2003; Knutson *et al.*, 2004; Du Preez *et al.*, 2005b).

The current study found a strong correlation between the size of the frog and the egg clutch size. In spite of a random selection, the R-R combination showed the largest female frogs producing more eggs. However, the percentage of eggs that hatched does not depend on the number of eggs laid, since 25-R combination yielded the highest percentage of 93% (fig. 3.2). Time to first Nieuwkoop and Faber (1967) development stage 66 ranged from 58 to 64 days. This is in line with Nieuwkoop *et al.* 1967 who reported that under controlled temperature conditions of 20°-25°, *X. laevis* tadpoles require approximately 58

days to complete metamorphosis. Development in all breeding combinations was fairly similar and the number of days to the last larvae reaching stage 66 was nearly the same for all combinations. Within the different breeding combinations, no significant difference was noted for the number of eggs laid, the percentage of eggs hatched, days to first stage 66 and days to last stage 66 metamorph.

The percentage survival within the different combinations was not significantly different although the R-R combination showed a rather low survival rate of 43.5%. However, there was no evidence of atrazine concentration response (see fig. 3.5). The sex ratio also showed no indication of concentration response (fig. 3.6). This is consistent with the presence of robust populations of frogs in association with atrazine use and crop production (Du Preez *et al.*, 2005; Hayes *et al.*, 2003; Knutson *et al.*, 2004). The snout-vent length of all the frogs was more or less the same, even though they were collected in different sites - but the body mass varied significantly. Frogs from site E had an average gonado somatic index which was high compared to those from the other sites. These observations indicate that the SVL, body mass and gonado somatic index are dependant on each other since they are all the highest in frogs collected from this site, and conversely, all those properties are smallest in frogs from site B. Testes lengths also vary within the site with frogs from site C having an average longer testis than the frogs from other sites.

Clawed frogs are renowned for being hardy animals and opportunistic breeders, and it is known that they utilise a wide variety of aquatic habitats. Du Preez *et al.* (2005) reported that Clawed frog females do not have synchronised ovulation and that at any given time of year some females will contain mature oocytes. Since the discovery of Shapiro and Zwarenstein (1934) that a subcutaneous injection of gonadotropin into a gravid female Clawed frog will

induce spawning, the use of Clawed frogs in embryological studies became very popular and the Clawed frog is probably - apart from the mouse and the chicken - the most studied laboratory animal.

4.2. Testicular ovarian folicles

One of the main objectives of this study was to determine whether different clades of *X. laevis* exist and whether they display the same prevalence of testicular ovarian follicles. The results show the existence of testicular ovarian follicles from specimens collected from northern sites. Testicular ovarian follicles were not detected in any of the southern sites. The results also show that frogs collected from sites A, B and C had regressed ovarian follicles and those from sites A, B and D had mature ovarian follicles. The mean number of regressed ovarian follicles were highest in frogs from site A and lowest in those from site B. The mean number of mature ovarian follicles were lowest in frogs from site B and highest in those from site C.

Testicular ovarian follicles were found in all breeding combinations, indicating that ovarian follicle presence shows no concentration response to atrazine. This observation is consistent with several other laboratory and field studies in *X. laevis* (Coady *et al.*, 2005; Du Preez *et al.*, 2005b; Hecker *et al.*, 2004; Hecker *et al.,* 2005a; Hecker *et al.*, 2005b; Smit *et al.*, 2005) and other frogs (Coady *et al.*, 2004; Reeder *et al.*, 2005). Therefore, there was no evidence proving that there are transgenerational effects of atrazine on spawning success or reproductive development of *X. laevis,* as recorded by Du Preez *et al.* (2005b), as robust populations of *X. laevis* are present in areas where exposures of atrazine have been observed and where it has been used in crop production for several decades.

4.3. Pesticides

All sites were screened for organochlorines, organophosphates, pyrethroids, PCBs and triazines. The only positive detection was in site G (Klapmuts, Bellville) in the Western Cape where atrazine (0.1 µg/L) simazine (0.4 µg/L) and teruthylazine (0.3µg/L) were detected.

Initially the aim was to collect at least 50 male frogs from each site, but in some areas that was not possible. Although *X. laevis* occurs widely throughout South Africa, they are not present in large numbers wherever they do. This was firmly established during the course of this study when we had difficulty collecting the required quota at each of the sites. The observed differences in coloration between frogs collected from northern and southern sites may be important, but a great deal more studies - including molecular and acoustic studies - will have to be undertaken to verify this. Du Preez *et al.* (submitted, Appendix B) conducted a molecular study and suggested that, based on mtDNA, there are at least two divergent clades of *X. laevis* in South Africa and that these two clades are separated by the Cape Fold Mountains. This is supported by Measey and Channing (2003).

The ages of frogs collected ranged from one to six years. The age profile also varied in the different sites. Differences observed between sites could be explained by the unique features of each site that would impact on the breeding behaviour.

Chapter 5

References

Chapter 5

REFERENCES

ALFORD, R.A. & RICHARDS, S.J. 1999. Global Amphibian declines a problem in ecology. *Annual Review of Ecology and Systematics,* 30: 133-165.

BELL, B.D., CARVER, S., MITCHELL, N.J.& PLEDGER, S. 2004. Infection of an invasive frog by the amphibian chytrid fungus. *Biological Conservation,* 120: 189-199.

BLACKER, A. W., FISCHBERG, M. & NEWTH, D. R. 1965. Hybridization of two subspecies of *Xenopus laevis* (Daudin). *Revue Suisse de Zoologie,* 72: 841-857.

BLACKER, A. W. & FISCHBERG, M. 1968. Hybridization of *Xenopus laevis petersi/poweri* and *X. laevis. Revue Suisse de Zoologie,* 75: 1023-1103.

BLAUSTEIN, A.R., ROMANSIC, J.M., KIESECKER, J.M. & HATCH, A.C. 2003. Ultraviolet radiation, toxic chemicals and amphibian population declines. *Diversity and Distribution,* 9: 123-140.

BLAUSTEIN, A.R. & KIESECKER, J.M. 2002. Complexity in conservation: Lessons from the global decline of amphibian populations. *Ecology Letters,* 5: 597-608.

BRITSON, C.A. & THRELKELD, S.T. 2000. Abundance metamorphosis, developmental, and behavioral abnormalities in *Hyla chrysoscelis* tadpoles following exposure to three agrichemicals. *Bulletin of Environmental Contamination and Toxicology,* 61: 154 -161.

BURTON, T.M. & LICKENS, G.E. 1975. Amphibians as models for studying environmental change.http//www.google.com.

CALBORN, T. 1998. Environmental Advocates of New York Endocrine disruption from environmental toxicants. *Environmental and Occupational Medicine,* 75: 198-207.

CARR, J.A., GENTLES, A., SMITH, E.E., GOLEMAN, W.L., URQUIDI, L.J., THUELT, K., KENDALL, R.J., GIESY, J.P., GROSS, T.S., SOLOMON, K.K. & VAN DER KRAAK, G.J. 2003. Response of larval *Xenopus laevis* to atrazine : assessment of gonadal and laryngeal morphology. *Environmental Toxicology and Chemistry,* 22: 396-405.

COADY, K.K., MURPHY, M.B., VILLENEUVE, D.L., HECKER, M., CARR, J.A., SOLOMON, K.R., SMITH, E.E., VAN DER KRAAK, G., 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. Ecotoxicology Environmental Safety,* 62: 160-173

COADY, K.K., MURPHY, M.B., VILLENEUVE, D.L., HECKER, M., JONES, P.D., CARR, J.A., SOLOMON, K.R., 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*). *Journal of Toxicology and Environmental Health,* 67: 941-957.

COOPER, R.L., STOKER, T.E., TYREY, L., GOLDMAN, J.M., & MC ELVOY, W.K. 2000. Atrazine disrupts the hypothalamic control of pituitary ovarian function. *Toxicological Sciences,* 53: 297-307

COLLINS, P. & STORFER, A. 2003. Global amphibian declines: sorting the hypotheses. *Diversity and Distributions,* 9: 89-98

CRAIN, D.A., GUILLETTE, L.J.JR., ROONEY, A.A., & PICKFORD, D.B. 1997. Alterations in steroidogenes in alligators (Alligator mississippiensis) exposed naturally and experimentally to environmental contaminants. *Environmental Health Perspectives,* 105: 528-533

DAWSON, D.A.& BANTLE, J.A. 1987. Development of a reconstituted water medium and preliminary validation of the frog embryo teratogenesis assay-*Xenopus* (FETAX). *Journal of Applied Toxicology,* 7: 237-224.

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. *Environmental Pollution,* 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 in South Africa. *African Journal of Herpetology,* 54: 61-68.

DU PREEZ L.H. 1996. A field guide to the frogs and toads of the Free State. Department of Zoology and Entomology, University of the Orange Free State, Bloemfontein, South Africa.

DU PREEZ, L.H., KUNENE, N., EVERSON, G.J., CARR, J.A., GIESY, J.P., GROSS, T.S.,HOSMER A.J.,KENDALL R.J., SMITH, E.E., SOLOMON K.R. & VAN DER KRAAK, G.J., 2008. Reproduction, larval growth, and reproductive development in African clawed frogs, (*Xenopus laevis*) exposed to atrazine. *Chemosphere,* 71: 546-552.

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.L. & WELDON, C., 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: 61-68.

DU PREEZ, L.H.,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., 2005. Seasonal exposures to triazine and other pesticides in surface waters in the western Highveld corn-production region in South Africa. *Environmental Pollution,* 135:131-141.

EISENREICH S., SCHOTTLER S. & HINES N. 1994. Standard Operating Procedure for isolation, Extraction and Analysisof Atrazine, DEA and DIA. Department of Environmental Sciences Rutgers University.Pp: 245-251.

EISENREICH, S., SCHOTTLER, S. & HINES, N. 1994. Department of Environmental Sciences Rutgers University, New Brunswick, N.J, USA. 245-251.

EVANS, B.J., KELLEY, D.B., TINSLEY, R.C., MELNICK, D.J. & CANNATELLA,D.C. 2004. A mitochondrial DNA phylogeny on African clawed frogs; Phylogeography and implications for polyploid evolution. *Molecular Phylogeny Evolution*, 33: 197-213.

EVANS, B.J., KELLEY, D.B., MELNICK, D.J. & CANNATELLA, D.C.2005. Evolution of RAG/1in polyploid clawed frogs. *Molecular Biology and Evolution,* 22: 193-1207.

EVANS, B.J., MORALES, J.C., PICKER, M.D., KELLEY, D.B. & MELMICK, D.J. 1997. Comparative molecular phylogeography of two *Xenopus* species, X. Gilli & X. *laevis,* in the South Western Cape province, South Africa. *Molecular Ecology,* 6: 333-342.

GIDDINGS, J.M., ANDERSON, T.A., HALL, L.W., JR, KENDALL, R.J., RICHARDS, R.P., SOLOMON, K.R. & WILLIAMS, W.M. 2005. A Probablistic Aquatic Ecological Risk Assessment of Atrazine in North American Surface Waters. SETAC Press, Pensacola, FL, USA.

GARDNER, T., 2001. Declining amphibian populations: A global phenomenon in Conservation Biology. *Annual Biodiversity and Conservation*, 24: 2: 25-44.

GROHOVAZ, G.S., HARLEY, E. & FABIAN, B. 1996. Significant mitochondrial DNA sequence divergence in natural populations of *Xenopus laevis* (pipidae) from South Africa. *Herpetologica*, 52: 247-253.

HAYES, T.B., COLLINS, A., MENDOZA, M., NORIEGA, M.,STUART, A. A. & VONK, A., 2003. Atrazine induced hermaphroditism at 0.1 ppb in American leopard frogs *(Rana Pipiens):* laboratory and field evidence. *Environmental Health Perspectives* III, 568-575.

HAYES, T.B. 2004. There 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., MENDONZA, M., NORIEGA, N., STUART, A.A. & VONK, A. 2002. Hermaphroditic, demasculinized frogs exposure to the herbicide atrazine at low ecology relevant doses. *Proceedings of the National Academy of Sciences, USA,* 99: 5476-5480.

HAYES, T.B., HASTON, K., TSUI, M., HOANG, A., HAEFFELE, C. & VONK, A. 2003. Atrazine-induced hermaphroditism at 0.1 ppd in American leopard frogs : Laboratory and field evidence. *Environmental Health Perspectives* III, 568-575.

HAYES, T.B., STUART, A.A., MENDONZA, 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 (17ß-estradiol): Support for the demasculinization / feminization hypothesis. *Environmental Health Perspectives,114 Sup.* 1: 134-141.

HAYES, T.B, COLLINS, A., LEE, M., MENDOZA, M., NORIEGA, N., STUART, A.A., & VONK, A. 2002a. Hermaphrodic, demasculinized frogs after exposure to herbicide atrazine at low ecologically relevant doses. *Proceedings of the NationalEP* Sciences, USA*, 419: 895-896.

HAYES, T.B, HARTON, K., TSUI, M., HOANG, A., HAEFFELE,C., & VONK, A. 2002b. feminization of male frogs in the wild. *Nature,* 419-896.

HAYES T.B., HASTON K., TSUI M., HOANG A., HAEFFELE C. & VONK A. 2002a. Atrazine-Induced hermaphroditism at 0.1 ppb in American Leopard frogs (Rana pipiens): laboratory and field evidence. *Environmental Health Perspectives,* 111: 568-575.

HECKER, M., MURPHY,M.B., COADY, K.K., VILLENEUVE,D.L., JONES, P.D., CARR, J.A., SOLOMON,K.R., SMITH, E.E., KRAAK, G. VAN DER ,GROSS, T., DU PREEZ, L.H., RONALD , R.J. & GIESY, J.P. 2006 Terminology of gonadal anomalies in fish and amphibians resulting from chemical exposures. *Environmental Contamination and Toxicology,* 187*:* 103-132

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 (*X. laevis*) from the corn-growing region of South Africa. *Environmental Toxicology and Chemistry,* 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.ER., 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. *Aquatic Toxicology,* 72: 383-396.

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*), 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 Sciences, Report Available from http://iccvam.niehs.nih.gov/methods/fetaxdoc/fetaxbrd.htm pp. 92.

IPSC international programme on Chemical Safety. 1990. Atrazine Health and Safety Guide N0.47, WHO Geneva.

IUCN species survival commission/Conservation International Centre for Applied Biodiversity Science / Biodiversity Assessment Unit.

JOBLING, S., NOLAN, M., TYLER, C.R., BRIGHTY, G. & SUMPTER, J.P. 1998. Widespread distribution in wild fish. *Environmental Science and Technology,* 32: 2498-2506.

JOOSTE, A.M. 2003 Evaluation of the effects of atrazine exposure on *Xenopus laevis* in South Africa. Environmental Sciences at the North-West University.

JOOSTE, A.M. & DU PREEZ, L.H. 2003. Microcosm study to evaluate the effect of atrazine exposures on African clawed frog (*Xenopus laevis*) tadpoles.Pesticides in non-target agricultural environments. Cape Town, South Africa.

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. *Environmental Science and Technology,* 39: 5255-52

KNUTSON, M.G., RICHARDSON, W.B., REINEKE, D.M., GRAY, B.R., PARMELEE, J.R. & WEICK, S.E.2004. Agricultural ponds support amphibian populations. *Ecological Applications,*14: 699-684.

LARSON, D.L., MCDONALD, A.J., FIVIZZANI, W.E., NEWTON & HAMILTON, S.J. 1998. Effects of the herbicide atrazine on *Ambystoma tigrinum* metarmorphosis: during larval growth and hormonal response. *Physiological zoology,* 71: 671-679.

LIPS, K.R., MENDELSON, J.R.,MUNOZ-ALONSO,A.,CANSECO-MARQUEZ,L. & MULCAHY,D.G. 2004. Amphibian population declines in Montane southern Mexico resurveys of historical localities. *Biological Conservation,* 119: 555-564.

MACKENZIE, C. A., BERRILL, M., METCALFE, C. & PAULI, B. D. 2003. Gonadal differentiation in frogs exposed to estrogenic and antiestrogenic compounds. *Environmental Toxicology and Chemistry,* 22: 2466-2475.

MEASEY, G.J. 1998 Diet of feral *Xenopus laevis* (Daudin) in South Wales, UK. *Journal of Zoology,* 246: 287-298.

MEASEY, G.J. & CHANNING, A. 2003. Phylogoegraphy of the genus *Xenopus* in southern Africa. *Amphibia-reptilia,* 24: 321-330.

MURPHY, M.B., HECKER, M., COADY, K.K., TOMPSETT, A.R., JONES, P.D., DU PREEZ, L.H., EVERSON, G.J.,SOLOMON, K.R., CARR, J.A., SMITH, E.E., KENDALL, R.J., VAN DER KRAAK, G.& GIESY J.P. 2006. Atrazine concentrations, gonadal grossmorphology and histology in ranid frogs was collected in Michigan agricultural areas. *Aquatic Toxicology*, 76 : 230-235.

NIEUWKOOP, P.O. & FABER, J.1967. Normal table of *X. laevis* (Daudin), 2nd edition. North Holland Publishing, North Holland, Amsterdam, the Nederlands.

OUELLET, M., BONIN, J., RODRIGUE, J., DESGRANGES, J.L. & LAIR, S.1997. Hindlimb deformities *(ectromelia, ectrodactyly)* in free-living anurans from agricultural habitats. *Journal of wild life Diseases,* 33 (1): 95-104.

POUNDS, J.A., CRUMP, M.L. 1994. Status and Trends of Amphibian Declines and Extinctions Worldwide. *Conservation Biology,* 8: 72.

POUNDS, J.A., FOGDEN, M.P.L. & CAMPBELL.J.H. 1997. Status and trends of Amphibian decline and extinctions worldwide. *Nature,* 11: 1307-1322.

POUNDS, J.A., FOGDEN, M.P.L. & CAMPBELL. J.H. 1999. Biological response to climate change on a tropical mountain. *Nature,* 398 (6728): 611-615.

REEDER , A.L., RUIZ, M.O., PESSIER, A., BROWN, L.E., LEVENGOOD, J.M., PHILIPS, C.A., WHEELER, M.B., WARNER, R.E. & BEASLEY, V.R. 2005. Intersexuality and the cricket frog decline: Historic and geographic trends. *Environmental Health Perspectives,* 113: 261-265.

REEDER A.L., G.L. FOLEY, D.K., NICHOLS, L.G., HANSEN, B., WIKOFF, S. FAEH, J. EISOLD, M.B., WHEELER, R., WARNER, J.E., MURPHY V.R. & BEASLEY. 1998. Forms and Prevalence of intersexuality and effects of environmental contaminations on sexuality in cricket frogs (*Acris crepitans*). *Environmental Health Perspectives,* 106: 261-266.

REEDER , A.L., RUIZ, M.O., PESSIER, A., BROWN, L.E., LEVENGOOD, J.M., PHILIPS, C.A., WHEELER, M.B., WARNER, R.E. BEASLEY, V.R., 2005. Intersexuality and the cricket frog decline: Historic and geographic trends. *Environmental Health Perspectives,* 119: 261-265.

SANDERSON, J.T., LETCHER,R.J., DRENTH,H.J. & BERG, M.VAN DEN. 2000. Development of in vitro bioassays to assess effects in enzymes involved in steroid synthesis and metabolism as mechanisms of endocrine disruption. *Organohalogen Compounds,* 49:326-329.

SANDERSON, J.T., SEINEN, W., GIESY, J.P. & VAN DEN BERG, M. 2002. 2-chloro-s-triazine herbicides induce aromatase activity in H295R human adrenal cortical carcinoma cells. A novel mechanism for astrogenicity. *Toxicological Sci*ences, 54: 121-127.

SANDERSON, J.T., LETCHER, R.J., HENEWEER, M., GIESY, J.P., VAN DEN BERG, M. 2001 Effects of chloro-s-triazine herbicides and metabolites on aromatase activity in various human cell lines and on vitellogenin production in male carp hepatocy. *Environmental Health Perspectives,* 109: 1 – 6.

SHAPIRO, H.A. & ZWARENSTEIN, H. 1934. A rapid test for pregnancy on *X. laevis*. *Nature*, 133: 762.

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. *African Journal of Herpetology,* 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. *Environmental Toxicology and Chemistry,* 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. Ecotoxicologial risk assessment of atrazine in amphibians. In: J.M. Clark Ohkawa, H. (Eds.). Environmental Fate and Safety Management

of Agrochemical. ACS Symposium Series No. 889. Vol. 2 American Chemical Society, Washington, DC, USA, 124-137 p.

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

STUART, S.N., CHANSON, J.S., COX, N.A., YOUNG, B.E., RODRIGUES, A.S.L., FISHMAN, D.L. & WALLER, R.W. 2004. Status and trends of Amphibians declined and extinctions worldwide. *Science,* 306 :1783-1786.

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 testes. *Environ. Toxicol. Chem.* 21, 527-531

TAVERA-MENDOZA, L.E. 2001. Influences of atrazine on gonadal differentiation in *Xenopus laevis* tadpoles during metemorphosis. Department of Biology. Montreal, PQ, Canada, Concordia University: 73 p.

TINSLEY, R.C. & MCCOID, M.J. 1996. The biology of *Xenopus* (Eds. Tinsley, R.C. & Kobel, H.R.) Clarendon Press, Oxford, UK. 81-94 p.

TINSLEY, R.C., LOUMONT, C. & KOBEL, H.R.1996. The biology of *Xenopus* (Eds. Tinsley, R.C. & Kobel, H.R.) Clarendon Press, Oxford, UK. 35-59 p.

U.S. EPA. 2001. United States Environmental Protection agency. ATRAZINE. HED,s Revised preliminary human health risk assessment for the registration eligibility decision (RED). Health effects division, Office of pesticide programs. Washington, DC, USA, Report No, PC Code: 080803, (January 19, 2001).

http://www.epa.gov/pesticides/reregistration/atrazine/revsd

VAN WYK, A.P. & DU PREEZ L.H. 1984. Voortplanting en ontvikkeling van die eiers by die platanna (*Xenopus sp.*) *Spectrum,* 22: 43-45.

WAKE, D.B. 1991. Declining amphibian populations. *Science*, 253:860.

WAKE, D.B. 2004. You Decide: Atrazine Ban. ACA's FROGS.ORG.

WELDON C. 1999. The sustainable utilization of the African Clawed Frog Xenopus laevis (Daudin). Department of Zoology and Entomology, University of the Free State, Bloemfontein, South Africa.

WELDON, C. 2002. Chytridiomycosis survey in South Africa. *Froglog,* 51:1 - 2

WELDON, C., DE VILLIERS, A.L. & DU PREEZ, L.H. 2007.Quantification of the African clawed frog trade from South Africa, with implications for biodiversity conservation. *African Journal Herpetology*, 56: 77-83

WICKS, B. 1998. Soil microbiology parameters as indicators of soil quality under improved fallow management system in Southwestern Nigeria plant soil. 202, 97-107.

WILBUR, H.M. & COLLINS, J.P. 1973. Ecological aspects of amphibian metamorphosis. *Science,* 182: 1305-1314.

WITSCHI, E. 1929. Studies on sex differentiation and sex determination in amphibians. III. Rudimentary hermaphrodites and Y chromosome in *Rana temporaria. Journal of Experimental Zoology,* 543: 157-222.

WITSCHI, E.1930. Studies on sex differentiation and sex determination in amphibians. *Journal of Experimental Zoology,* 56: 149-165.

WITSCHI, E. 1942.Temperature factors in the development and the evolution of sex. *Biological Symposia,* 6: 51-70.

WYMAN, R.L. 1990. What's Happening to amphibians? *Conservation Biology,* 4: 350-352.

YU, M-H. 2000. Impacts of Environmental Toxicants on Living Systems. *Environmental Toxicology.*

Appendix A

MANUSCRIPT SUBMITTED

Population-specific incidence of testicular ovarian follicles in *Xenopus laevis* from South Africa: A potential issue in endocrine testing

Louis H. Du Preez, Nisile Kunene, Robert Hanner, Ben J. Evans, John P. Giesy, Keith R. Solomon, Alan Hosmer & Glen J. Van Der Kraak

Population-specific incidence of testicular ovarian follicles in *Xenopus laevis* from South Africa: A potential issue in endocrine testing

Louis H. Du Preez[*], Nisile Kunene[*], Robert Hanner[§], Ben J. Evans[†], John P. Giesy[‡], Keith R. Solomon[⊥], Alan Hosmer[λ] & Glen J. Van Der Kraak[§]

[*] School of Environmental Sciences and Development, North-West University, Potchefstroom 2531, South Africa.

[†] Department of Biology, McMaster University, Hamilton, ON., Canada L8S 4K1.

[‡] Department of Veterinary Biomedical Sciences, University of Saskatchewan, Saskatoon, Saskatchewan, Canada, Department of Biology and Chemistry, City University of Hong Kong, Tat Chee Avenue, Kowloon, Hong Kong, SAR, China, and National Food Safety and Toxicology Center, Zoology Department, and Centre for Integrative Toxicology, Michigan State University, East Lansing, Michigan 48824.

[§] Department of Integrative Biology, University of Guelph, Guelph, ON, Canada, N1G 2W1.

[λ] Syngenta Crop Protection, Greensboro, NC, 27419-8300, USA.

[⊥] Centre for Toxicology and Department of Environmental Biology, University of Guelph, Guelph, ON, Canada, N1G 2W1.

Summary

The African clawed frog (*Xenopus laevis*) is widely used as a laboratory model and is scheduled to be a primary test organism in recently mandated testing of existing chemicals in both North America and Europe. There has been controversy over the possible effects of the widely-used herbicide, atrazine, on gonadal development in amphibians, including its possible role in promoting a form of abnormality in the testes characterized by the presence of ovarian follicles (oocytes) within the testis of *X. laevis*. We analyzed adult *X. laevis* collected along a transect running from the Southwest Western Cape region (W Cape) to the Northeast (NE) of South Africa and found that,

irrespective of exposure to atrazine, male *X. laevis* from NE sites contained testicular ovarian follicles whereas none were observed in male *X. laevis* from the W Cape where atrazine is not widely used. Phylogenetic analysis of mitochondrial and nuclear genes indicates that frogs from the W Cape are evolutionarily divergent from those from NE South Africa and the rest of sub-Saharan Africa. This suggests that the occurrence of testicular ovarian follicles is a natural phenomenon that varies in frequency among evolutionarily and geographically distinct populations of *X. laevis*, and that this is a physiological/developmental difference between them. Compared to other NE populations where atrazine is neither used nor detected, we did not observe an increased incidence of testicular ovarian follicles in the NE portion of our transect where atrazine is used and where exposure was detected. These findings provide a possible explanation for why conflicting results have been reported concerning the impact of atrazine on amphibian sexual differentiation and highlight the importance of understanding taxonomic status of the experimental animal. Even in common laboratory animals, there is a need for their correct taxonomic characterization before their use in tests for endocrine disruption.

.....

Testicular ovarian follicles (TOFs, also reported in the literature as testicular oocytes; TOs) are a phenomenon that can be induced by exposure to estrogens such as 17β-estradiol and which have been suggested as a potential endpoint for characterizing exposure to estrogenic compounds [1-3]. TOFs are female reproductive cells with an intact nucleus, nucleoli, and a surrounding squamous epithelial layer embedded in testis tissue [3] and can occur naturally but to varying degrees in the testes of some frog species [4-7]. Results of studies on exposure to atrazine and its linkage to the incidence of TOFs fall into two categories. Some researchers report no incidence in unexposed animals but increased incidence of TOFs in developing larval frogs exposed to concentrations of ≥ 0.1 µg atrazine/L [8]. Others report the presence of testicular oocytes in unexposed as well as atrazine-exposed frogs in laboratory and field studies with no relationship to exposure concentration [9-12]. In the grey tree frog, *Acris crepitrans*, TOFs

were observed in specimens collected both before and after the introduction of atrazine to the market [7]. The number of TOFs has been reported to decrease with age in *X. laevis* [11,13] and regressed TOFs are more frequently observed in older animals [11].

For many years, exports of *X. laevis* as laboratory animals took place from the W Cape area of South Africa [14], but the origins of the many colonies of *X. laevis* in laboratories around the world are, for the most part, unknown. The taxonomic relationships among all species of *Xenopus*, including morphologically differentiated populations of *X. laevis*, have been characterized by both mitochondrial (mtDNA) and nuclear DNA [15-19] and multiple subspecies of *X. laevis* have been identified [20]. Based on mtDNA, there are at least three divergent lineages in *X. laevis* within South Africa (SA) [18]. The distribution of these mtDNA lineages is potentially consistent with a role played by the Cape Fold Mountains (Figure 1), which separate the southern winter-rainfall and northern summer-rainfall areas of SA, in restricting gene flow within *X. laevis*. However, relationships between these mtDNA lineages and the incidence of TOFs or molecular variation in nuclear DNA have not been investigated.

We explored morphology, molecular diversity, and the incidence of TOFs in *X. laevis* individuals that were collected in habitats in SA with little or no atrazine exposure, ranging from downwind and near to the major atrazine use-area in maize crops to areas that are upwind, far from this region, and that have no atrazine exposure – current or historical (Sites A–I, Figure 1). DNA sequences were obtained from representative individuals from these localities and from additional samples from throughout sub-Saharan Africa. We included other South African species (*X. gilli* and *X. muelleri*) in our molecular analysis and used *S. tropicalis* as an outgroup. In addition to these wild-collected frogs, we analyzed mtDNA from *X. laevis* from *Xenopus*-1 Inc. and *Xenopus* Express who supply this species to the north-American market and maintain colonies developed from *X. laevis* originally from suppliers in the W Cape [21].

At each site in SA (except Sites A and H), the catchment was characterized for land use and water and sediment samples were taken for analysis (see SI). Organochlorine,

organophosphorus, and pyrethroid pesticides were not detected at any of these locations (see Methods). Atrazine, its metabolites, and other triazines also were not detected (see Methods), except at Site I, where low concentrations of atrazine, simazine, and terbuthylazine were observed (0.1, 0.4, and 0.3 µg/L, respectively).

Molecular variation is geographically structured in *X.* laevis (Figure 3). Within SA, at least two divergent populations exist, one in the W Cape at Sites A, B, C, and D and the other in NE SA at Sites F, G, H, and I, and there is a contact zone or cline between them at Site E. Over the entire range of *X. laevis*, four diverged mtDNA lineages were observed and were carried by (a) individuals from Sites A, B, C, D, and six individuals from site E, (b) four individuals at Sites E and all from Site F, (c) individuals from Sites G, H, I and Malawi, and (d) individuals from the rest of the sub-Saharan samples (Botswana, Tanzania, Rwanda, Uganda, DRC, Congo Brazzaville, Cameroon, and Nigeria). The recombination activating gene 2 (RAG2) locus also has unique alleles in each of these four general regions and admixed variation at Site E is revealed by heterozygous individuals. The geographic distribution of variation in the hypervariable region of the androgen receptor (AR) locus is similar to RAG2, including heterozygous individuals at Site E, but with the exception that AR alleles at site F and some individuals at Site E were identical to those found at Sites G, H, and I in NE SA.

Site E is either a point of secondary contact or a cline between a population in the W Cape (Sites A, B, C, and D) and a population in the rest of SA (Sites F, G, H, and I) plus Malawi. Six mtDNA haplotypes from Site E are closely related to those from the W Cape and four are closely related to those from Site F (Figure 3). Of nine genotypes at RAG2 and AR, none were homozygous at both loci for alleles from only one of these populations, one individual was homozygous at each locus for alleles from different populations, and the others were heterozygous at one (one individual) or both loci (7 individuals) for alleles from both of these populations (Figure 3). This indicates that these populations are reproductively compatible in nature. All commercially purchased individuals that we analyzed appear to be derived from the W Cape population in that

their mtDNA haplotypes and alleles at both nuclear loci cluster with wild-caught individuals sampled in this region.

There were morphological differences between the two major populations of frogs in SA and between sites within these regions. Male frogs collected in the W Cape region (Sites B, C, and D) were significantly heavier, were longer, and had greater testis mass than those from the NE region (Sites E, F, G, and I; Figure 2A, 2B, and SI Figure 4, respectively, $p = <0.001$ Mann-Whitney Rank Sum Test) but there were also significant differences among sites within this region (see SI). This is consistent with results of another study where intra- and inter-site variation was observed [22]. Median gonadosomatic index (GSI - the weight of the testes as a percentage of the total body weight) of male *X. laevis* from W Cape region was significantly greater than those from NE region ($p = <0.001$ Mann-Whitney Rank Sum Test, Figure 2C). The masses of testes of male *X. laevis* at Sites G and D were significantly less than at other sites (see SI), resulting in small GSIs at these sites. Ages of frogs ranged from one to six years with a median age of 2 yr in the NE region and 1 yr at the W Cape region. Frogs from the NE region were significantly older than those from the W Cape region ($p = <0.001$ Mann-Whitney Rank Sum Test, see SI Figure 2, Table 2), due primarily to the values of Site E, which had more older *X. laevis* and where one-year old frogs comprised only 12% of the sample (see SI).

TOFs were observed in frogs from the mostly atrazine-free NE sites (Sites E, F, G, and I) but none were observed at the W Cape sites (Sites B, C, and D). The total number of mature and regressed TOFs per individual varied among NE sites (Figure 5), although not significantly ($p = >0.05$, Mann-Whitney Rank Sum Test). Previous studies have reported that older frogs have fewer TOFs [11,13], which may explain the presence of only regressed TOFs in *X. laevis* from Site E, where frogs were significantly older (see SI). These data, taken together with molecular divergence in mitochondrial and nuclear DNA, highlight geographic variation with respect to natural occurrence of TOFs in the absence of atrazine. The incidence of TOFs at Site I, where atrazine was detected, was not greater than incidence at the other atrazine-free NE sites. This is consistent with

other observations on the effects of atrazine in *X. laevis* in both laboratory [9] and field studies [10,11,13] and does not support an association between atrazine exposures and the incidence of TOFs in the portion of the range of *X. laevis* that is NE of the Cape Fold Mountains. Our findings also support the observations of others [4-7] that the occurrence of TOFs can be a natural phenomenon, and that its prevalence varies between species or populations, even those that are closely related. Because atrazine was not detected in sites from the W Cape, it was not possible to test for a relationship between incidence of TOFs in adults in this population and atrazine exposures in the field. However, TOFs have not been reported in recent laboratory studies on frogs derived from this area and exposed to concentrations of atrazine between 0.1 and 100 µg/L from tadpole (Nieuwkoop and Faber stage 46) to completion of metamorphosis [23].

The absence of naturally occurring TOFs from *X. laevis* in the W Cape region must be considered when assessing the significance of reproductive/developmental endpoints in *X. laevis*. There is developmental variation between *X. laevis* populations in different parts of its sub-Saharan range, and this variation corresponds with evolutionary divergence between them (this study) [15,18,19]. That recent exports of *X. laevis* from SA only take place from the W Cape and Southern Cape region [21] implies that current stock in the USA and other parts of the world is *X. laevis laevis*. This was confirmed by our multi-locus analyses of molecular variation in *X. laevis* obtained from the two major suppliers in the US. However, the provenance of older stocks is less clear and domesticated stocks potentially could even be derived from multiple geographic sources or their offspring. Moreover, laboratory crosses of different subspecies of *X. laevis* produce fertile and apparently normal progeny [24,25] and we detected evidence of genetic exchange between molecularly divergent populations at Site E. This may explain why studies in different laboratories have produced different results and behooves the genetic characterization of test animals before extrapolating conclusions.

Methods

Collection of test animals and residue analyses

Four to ten baited bucket *Xenopus* traps were set in the water bodies at the selected collection sites. Sediment and water samples were taken in the shallow water in the each of four quadrants of the pond. Water samples were pooled into two 1-L sub-samples collected in 1-L solvent rinsed (acetone and hexane) glass bottles. Water samples were stored at 4°C [26] until analysis of atrazine and terbuthylazine and chloro-metabolites in environmental samples, as well as other pesticides. Analyses for triazines and metabolites at Sites B, C, D, E, F, and G were conducted with a Method Detection Limit (MDL) of 0.025 µg/L by Dr. Robert Yokley (Syngenta Laboratories). Pesticide residues at Site I were determined in a previous study [27,28]. Other pesticides and elements in sediment and water were analyzed at a MDL of 0.1 µg/L by the South African Bureau of Standards, a certified laboratory located in Pretoria, SA. Water quality parameters were recorded (See SI).

Analyses of morphology, histology, and skeletochronology

Frogs were weighed (± 0.01g) and the snout-vent length measured. Malformations and other abnormal morphological characteristics were recorded. Frogs were then dissected, the gonads examined for testicular anomalies, measured, and photographed. One gonad was placed in a biopsy cassette and fixed in Bouin's fixative for 48 hours then transferred to 70% ethanol for storage. Preserved testicular tissue was embedded, sectioned, and stained as previously described [11] to produce serial sections (7 µm) of the entire testis. Every section was examined for the presence of TOFs. Age profile of specimens was determined by skeletochronology as previously described [27].

DNA sequencing and phylogenetic analysis – See Supplemental Information

---

References

1. Jobling, S., Nolan, M., Tyler, C. R., Brighty, G. & Sumpter, J. P. Widespread disruption in wild fish. *Environ. Sci. Technol.* **32**, 2498-2506 (1998).
2. Mackenzie, C. A., Berrill, M., Metcalfe, C. & Pauli, B. D. Gonadal differentiation in frogs exposed to estrogenic and antiestrogenic compounds. *Environ. Toxicol. Chem.* **22**, 2466-2475 (2003).

20. Tinsley, R. C., Loumont, C. & Kobel, H. R. in *The Biology of Xenopus* (eds. Tinsley, R. C. & Kobel, H. R.) 35–59 (Clarendon Press, Oxford, UK, 1996).
21. Weldon, C., De Villiers, A. L. & Du Preez, L. H. Quantification of the African clawed frog trade from South Africa, with implications for biodiversity conservation. *Afr. J. Herp.* **56**, 77-83 (2007).
22. Everson, G., Du Preez, H. H. & Solomon, K. R. Reproductive biology of the African clawed frog, *Xenopus laevis*: A histometric analysis. *Afr. J. Herp.* **Submitted** (2007).
23. USEPA. 321 (United States Environmental Protection Agency, Washington, DC, USA, 2007).
24. Blackler, A. W., Fischberg, M. & Newth, D. R. Hybridization of two subspecies of *Xenopus laevis* (Daudin). *Revue Suisse de Zoologie* **72**, 841-857 (1965).
25. Blackler, A. W. & Fischberg, M. Hybridization of *Xenopus laevis petersi/poweri* and *X. l. laevis*. *Revue Suisse de Zoologie* **75**, 1023-1103 (1968).
26. Eisenreich, S., Schottler, S. & Hines, N. 245–251 (Department of Environmental Sciences Rutgers University, New Brunswick, NJ, USA, 1994).
27. Du Preez, L. H. et al. 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 (2005).
28. Du Preez, L. H. et al. 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 (2005).

Supplemental information is available on.....

Achnowledgements

We thank Gideon Everson, North West University for assistance, Christa Maitland, Genome Canada (through the Ontario Genomics Institute), and the Canadian Centre for DNA Barcoding for access to facilities. Syngenta Crop Protection Inc. and the National Research and Engineering Council of Canada partially funded this study.

Correspondence and requests for materials should be sent to L.H.dP. (Louis.DuPreez@nwu.ac.za)

3. Hecker, M. et al. Terminology of gonadal anomalies in fish and amphibians resulting from chemical exposures. *Rev. Environ. Contam. Toxicol.* **187**, 103-132 (2006).
4. Witschi, E. Studies on sex differentiation and sex determination in amphibians. III. Rudimentary hermaphrodites and Y chromosome in *Rana temporaria*. *J. Exp. Zool.* **543**, 157-222 (1929).
5. Witschi, E. Studies on sex differentiation and sex determination in amphibians. *J. Exp. Zool.* **56**, 149-165 (1930).
6. Witschi, E. Temperature factors in the development and the evolution of sex. *Biol. Symposia.* **6**, 51-70 (1942).
7. Reeder, A. L. et al. Intersexuality and the cricket frog decline: Historic and geographic trends. *Environ. Health Perspect.* **113**, 261-265 (2005).
8. Hayes, T. B. et al. Atrazine-induced hermaphroditism at 0.1 ppb in American leopard frogs (*Rana pipiens*): Laboratory and field evidence. *Environ. Health Perspect.* **111**, 568-575 (2003).
9. Coady, K. K. et al. 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 (2005).
10. Smith, E. E. et al. 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 (2005).
11. Jooste, A. M. et al. Gonadal development of *Xenopus laevis* larvae exposed through larval development to atrazine in outdoor microcosms. *Environ. Sci. Technol.* **39**, 5255-5261 (2005).
12. Murphy, M. B. et al. Atrazine concentrations, gonadal gross morphology and histology in ranid frogs collected in Michigan agricultural areas. *Aquat. Toxicol.* **76**, 230-245 (2006).
13. Du Preez, L. H. et al. Reproduction, larval growth, and reproductive development in African clawed frogs, (*Xenopus laevis*) exposed to atrazine. *Chemosphere* **doi:10.1016/j.chemosphere.2007.09.051** (2007).
14. Tinsley, R. C. & McCoid, M. J. in *The Biology of Xenopus* (eds. Tinsley, R. C. & Kobel, H. R.) 81–94 (Clarendon Press, Oxford, UK, 1996).
15. Evans, B. J., Kelley, D. B., Tinsley, R. C., Melnick, D. J. & Cannatella, D. C. A mitochondrial DNA phylogeny of African clawed frogs: Phylogeography and implications for polyploid evolution. *Mol. Phylogen. Evol.* **33**, 197–213 (2004).
16. Evans, B. J., Morales, J. C., Picker, M. D., Kelley, D. B. & Melnick, D. J. Comparative molecular phylogeography of two *Xenopus* species, *X. gilli* and *X. laevis*, in the southwestern Cape Province, South Africa. *Mol. Ecol.* **6**, 333-343 (1997).
17. Evans, B. J., Kelley, D. B., Melnick, D. J. & Cannatella, D. C. Evolution of RAG-1 in polyploid clawed frogs. *Mol. Biol. Evol.* **22**, 193-1207 (2005).
18. Grohovaz, G. S., Harley, E. & Fabian, B. Signifcant mitochondrial DNA sequence divergence in natural populations of *Xenopus laevis* (Pipidae) from South Africa. *Herpetologica.* **52**, 247-253 (1996).
19. Measey, G. J. & Channing, A. Phylogeography of the genus *Xenopus* in southern Africa. *Amphibia-Reptilia* **24**, 321-330 (2003).

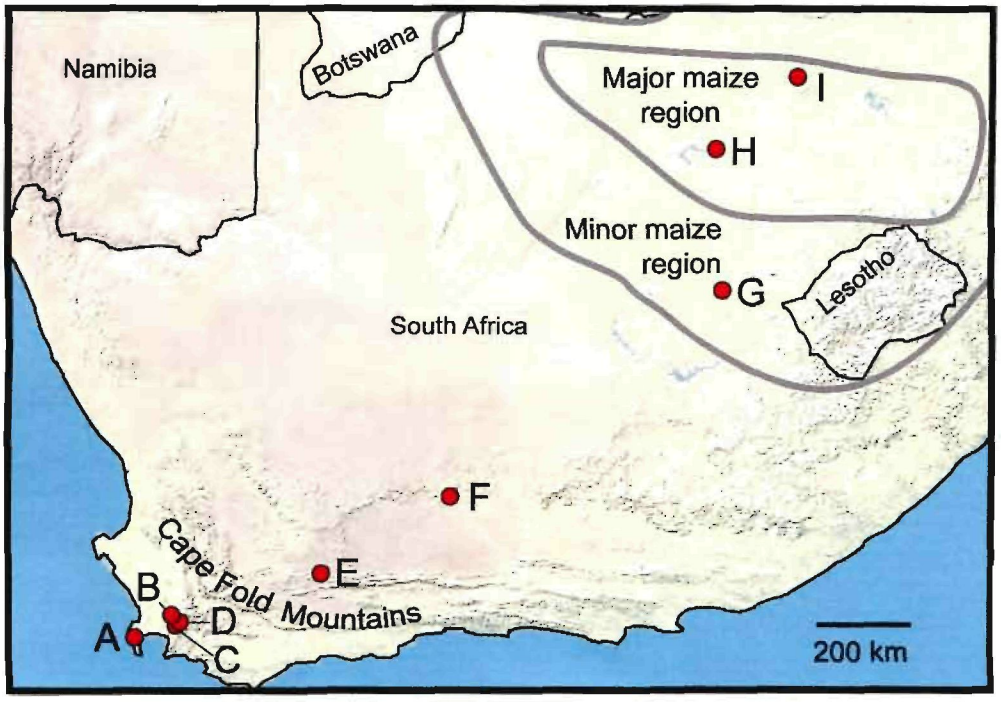


Figure 1. Map of South Africa with collection sites A-I, major, and minor maize regions, indicated. For more details on the sites, see SI.

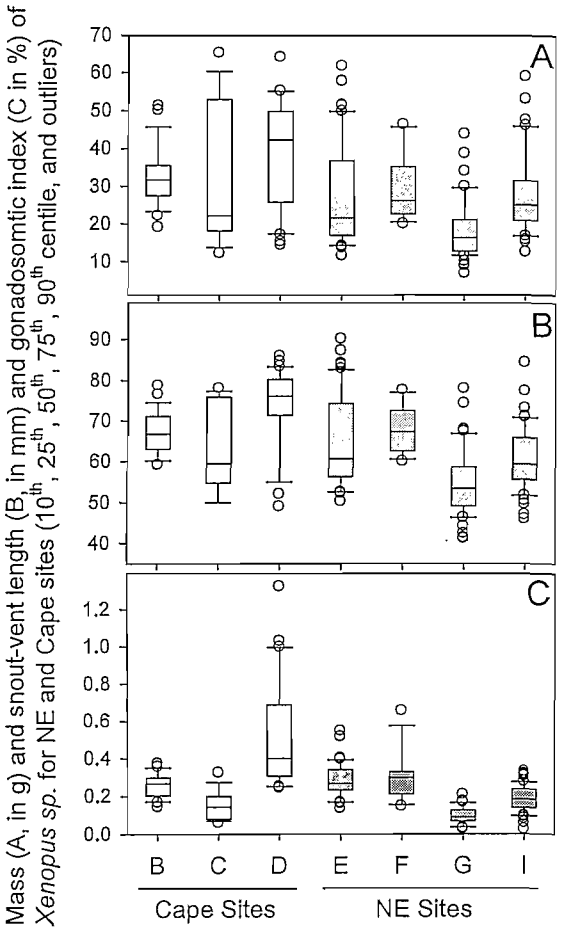


Figure 2. A) mass, B) snout-vent length, and C) gonado-somatic index of male *Xenopus laevis* from the collection sites. Measurements were not taken on frogs from Sites A or H, or other locations in sub-Saharan Africa.

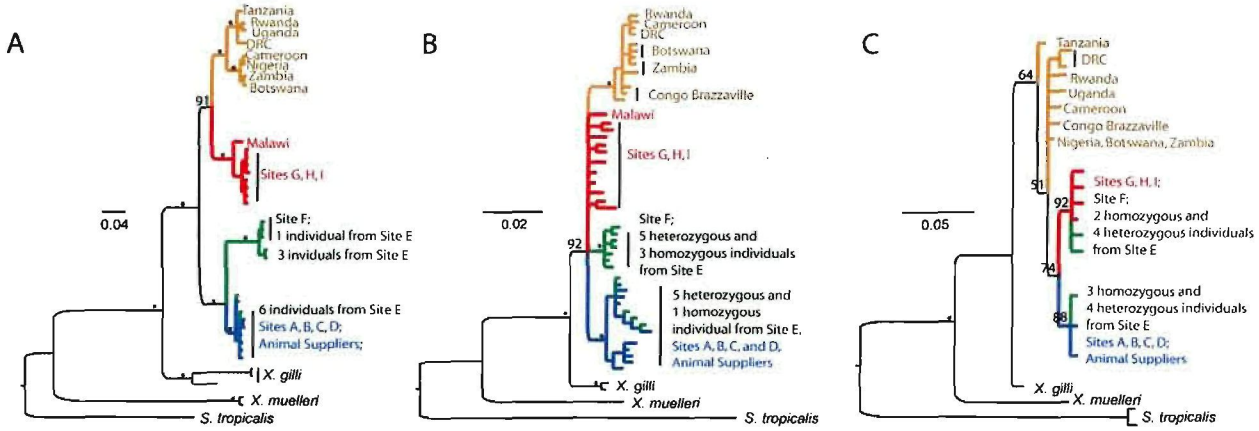


Figure 3. Bayesian consensus phylograms of relationships between unique haplotypes and alleles of (A) mitochondrial DNA (mtDNA), (B) the recombination activating gene 2 (RAG2), and (C) the hypervariable region of the androgen receptor (AR). A total of 87, 75, and 119 *Xenopus laevis* individuals, respectively, were sequenced for each of these genes. Branches with posterior probabilities of interest that are over 90 are labeled and those greater than 95% are indicated with an asterisk. Sampling sites refer to those depicted in Figure 1.

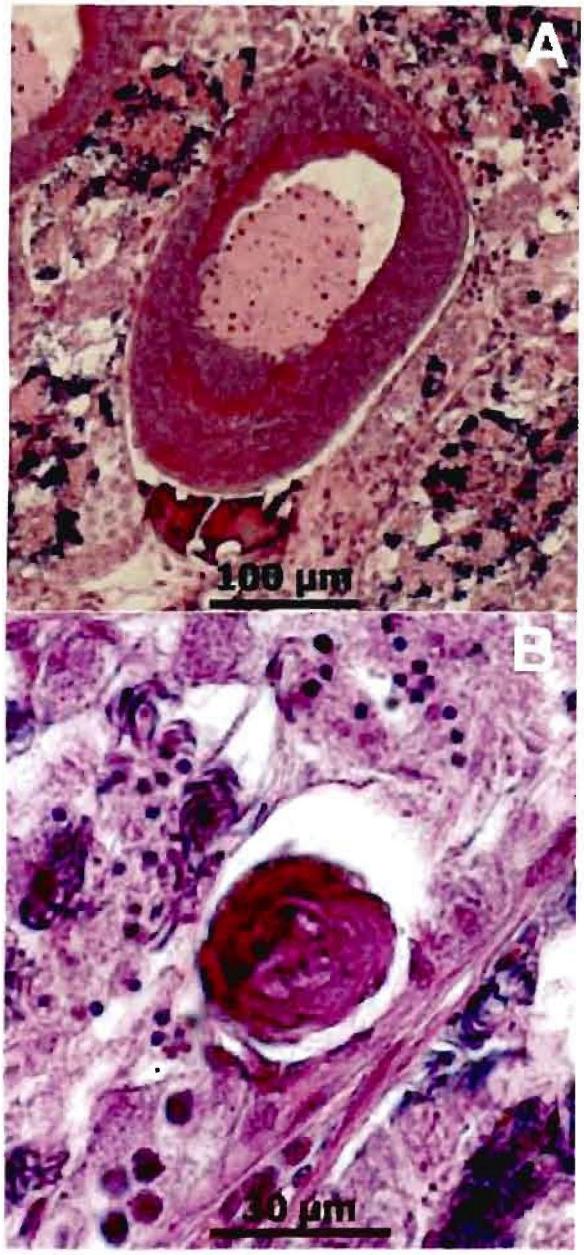


Figure 4. Testicular ovarian follicles. A), mature testicular ovarian follicle from a two-year old adult male *X. laevis* and B), a regressed testicular ovarian follicle from a four-year old adult male.

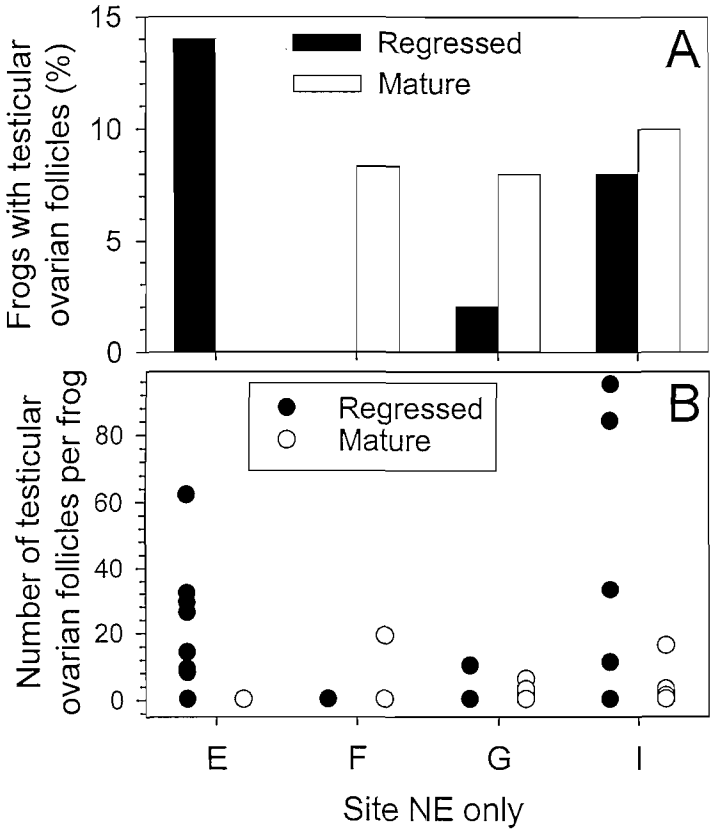
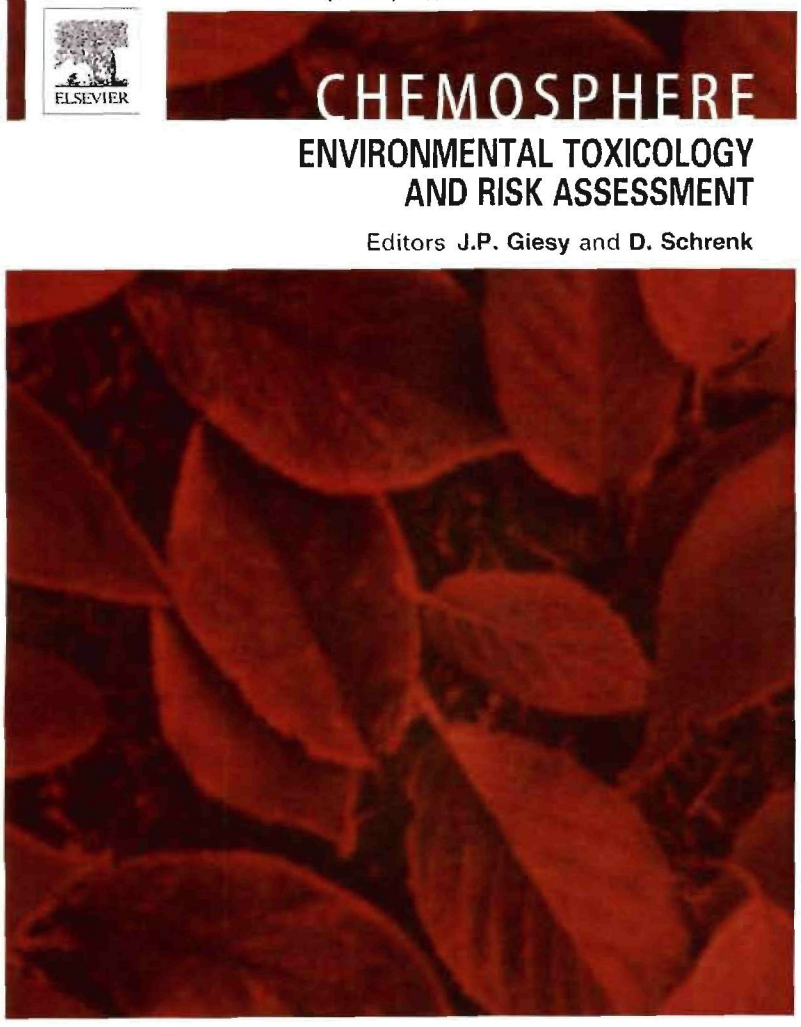


Figure 5. A) Prevalence of testicular ovarian follicles (TOFs) in *Xenopus laevis* from the NE sites. B) Numbers of TOFs per frog. Atrazine was detected at concentrations ≥ MDL (0.025 µg/L) at Site I only. Mature and regressed TOFs were not observed in frogs from the W Cape sites (Sites B, C, and D). Data on the incidence of TOFs were not obtained from frogs from Sites A or H, or other locations in sub-Saharan Africa..

Volume 71, issue 3, March 2008 ISSN 0045-6535



Available online at www.sciencedirect.com
ScienceDirect
Chemosphere 71 (2008) 546–552
CHEMOSPHERE
www.elsevier.com/locate/chemosphere

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*

Received 11 June 2007; received in revised form 7 September 2007; accepted 27 September 2007
Available online 14 November 2007

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.

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.,

* Corresponding author. Tel.: +11 27 18 299 2372; fax: +11 27 18 299 2370.
E-mail address: Louis.DuPreez@nwu.ac.za (L.H. Du Preez).

0045-6535/$ - see front matter © 2007 Elsevier Ltd. All rights reserved.
doi:10.1016/j.chemosphere.2007.09.051

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.

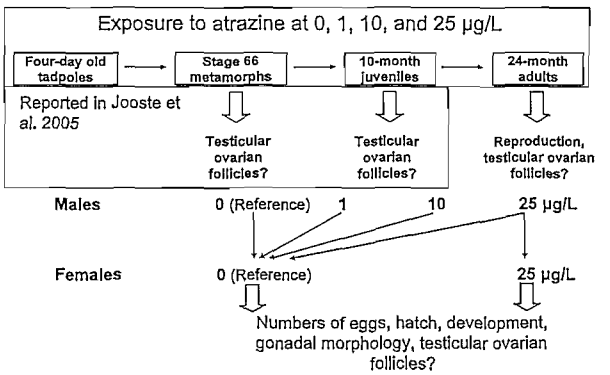


Fig. 1. Illustration of the experimental design and cross breeding.

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

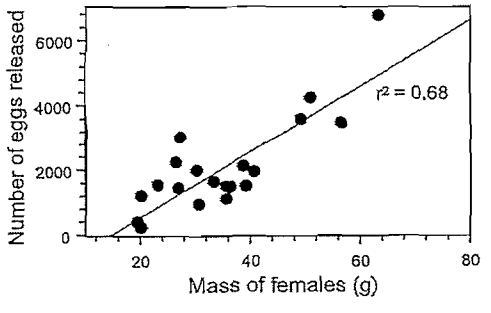


Fig. 2. Relationship between mass of F1 female frogs and number of eggs released.

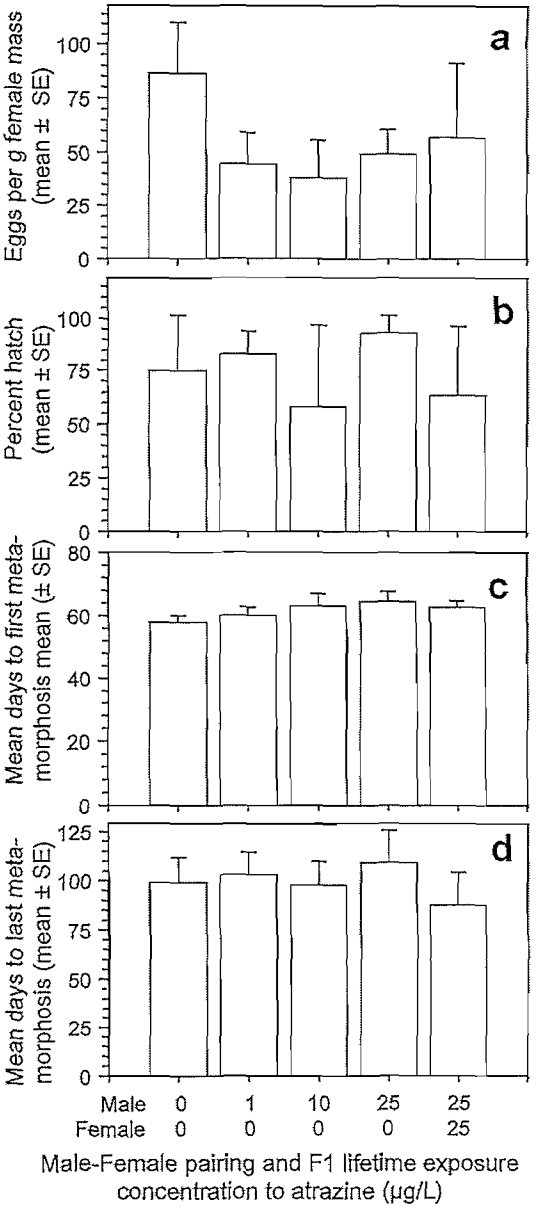


Fig. 3. a. Mass-corrected number of eggs released per F1 female. b. Percent hatch of F2 eggs. c. Days to reach stage 66 of the first F2 larva. d. Days to reach stage 66 of the last F2 larva. N = 4 pairs per treatment.

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

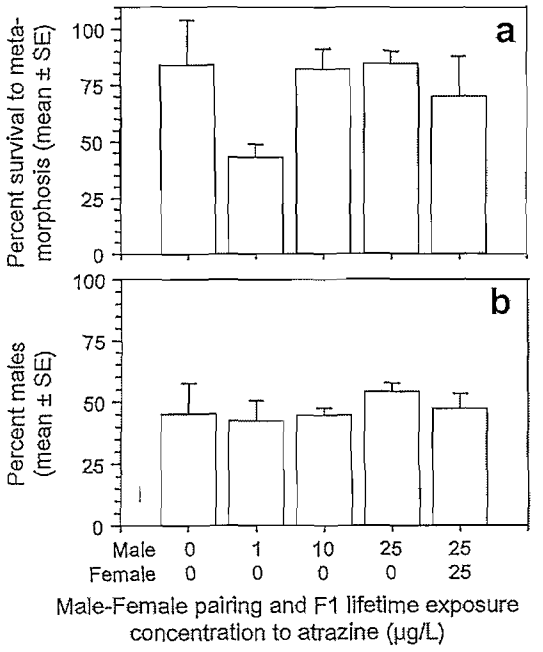


Fig. 4. a. Survival of F2 larvae at stage 66. b. Percent males. Percent survival based on N = 200, sex-ratio on number of survivors.

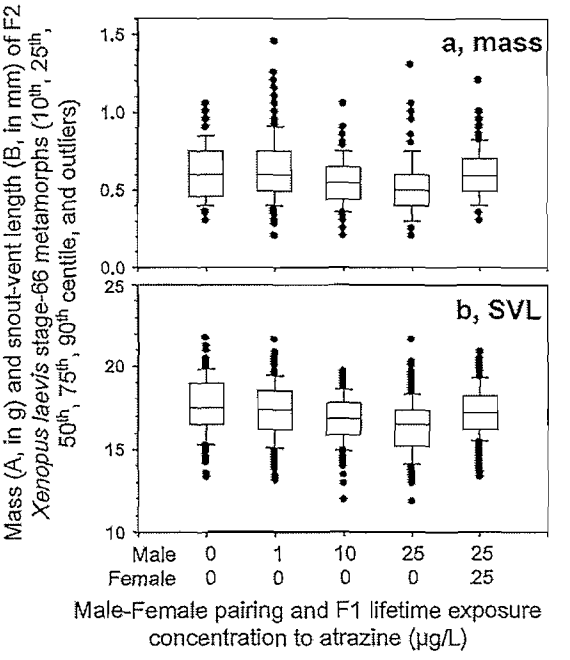


Fig. 5. a. Mass of F2 larvae at stage 66. b. Snout-Vent Length (SVL) of F2 larvae at stage 66. As data were not normally distributed, median, centiles, and outliers are show in a box-plot format. Data are the combined values for all the F2 progeny from the four pairs of adults and N ranged from 140 to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

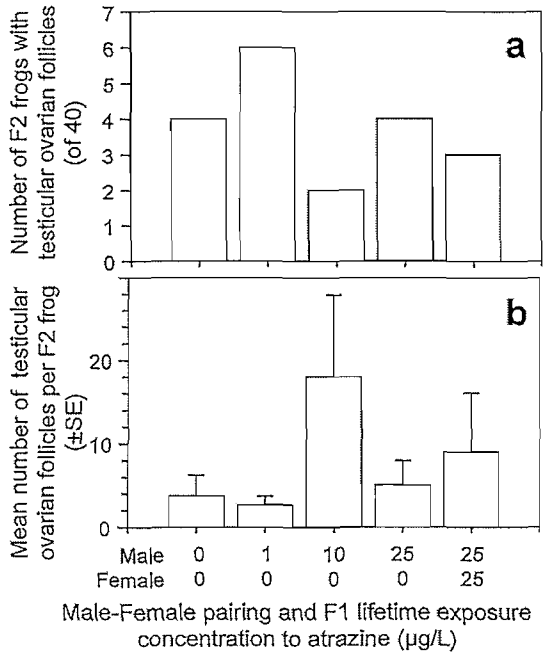


Fig. 6. a. Number of F2 frogs with testicular ovarian follicles. b. Number of testicular ovarian follicles in those F2 frogs with ovarian follicles in one or both testes. N ranged from 74 to 110.

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

This research was conducted under the oversight of the Atrazine Endocrine Ecological Risk Assessment Panel, Ecorisk, Inc., Ferndale, WA with a grant from Syngenta Crop Protection, Inc.

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.