

Original article

## Assessment of laryngeal muscle and testicular cell types in *Xenopus laevis* (Anura Pipidae) inhabiting maize and non-maize growing areas of South Africa

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**Abstract.**—We tested the hypothesis that adult African clawed frogs (*Xenopus laevis*) inhabiting water bodies in maize-growing areas (MGA) of South Africa would exhibit differences in testicular structure compared to frogs from water bodies in non-maize-growing areas (NMGA) in the same locale. Adults of both sexes were collected during the autumn of 2002 in South Africa, and stereological analytical techniques were used to quantify the distribution of testicular cell types. In addition, total laryngeal mass was used as a gauge of secondary sex differences in animals from MGA and NMGA study sites. Evaluation of the total laryngeal mass revealed that there were no statistically significant differences between *X. laevis* of the same sex from the NMGA and MGA sites. Mean percent fractional-volume values for seminiferous tubule distribution of testicular cell types of mature *X. laevis*, ranged from 3-4% for spermatogonia, 26-28% for spermatocytes, 54-57% for spermatozoa, and 14-15% for other cells types. The mean percent volume for blood vessels ranged from 0.3-0.4%. These values did not differ significantly between frogs from NMGA and MGA areas. Collectively, these data demonstrated no differences in gonadal and laryngeal development in *X. laevis* collected in South Africa from MGA and NMGA areas and that there is little evidence for an effect of agricultural chemicals used in maize production functioning as endocrine disrupters in this species. Screening of *X. laevis* testes revealed a small incidence of Stage 1 testicular oocytes in adult male frogs collected from the NMGA (3%) and MGA (2%).

**Key words.**—*Xenopus laevis*, stereology, spermatogenesis, atrazine, laryngeal muscle, reproduction.

Recent studies of mammals and reptiles suggest that atrazine may interfere with the endocrine regulation of reproduction, either through effects at the level of the hypothalamus (Cooper *et al.* 2000), or effects on gonadal aromatase activity (Crain *et al.* 1997; Crain *et al.* 1999; Sanderson *et al.* 2000, 2001). Recently,

Hayes *et al.* (2002b), reported that atrazine treatment (0.1-25 µg/L) under laboratory conditions decreased laryngeal size in male, but not female *X. laevis*, and increased the incidence of gonadal abnormalities when compared to unexposed controls. However, in a similar study, Carr *et al.* (2003) found no effect

of atrazine on laryngeal development in *X. laevis*, but reported a small percentage of gonadal abnormalities in atrazine-treated frogs. The incidence of gonadal abnormalities was only statistically different from controls at 25 µg atrazine/L. A study on Cricket Frogs, *Acris crepitans*, suggested a trend, although not statistically significant, between atrazine exposures and altered reproductive development (Reeder *et al.* 1998). However, in a temporal analysis of *A. crepitans*, the incidence of intersexual gonads suggests that exposures to substances other than atrazine may be responsible for the reported effects (Beasley *et al.* 2001). The effects of atrazine observed in laboratory tests with *X. laevis* have yet to be evaluated in exposed field populations. Furthermore, there is a lack of appropriate quantitative assessment tools that would minimise investigator bias in data collection.

Stereology is a method used to obtain quantitative information of structural changes in tissue and cells (Elias & Hyde 1980). Based on the existing literature, a stereological model that provides information on the distribution of testicular cell types of the seminiferous tubules, was developed for the *X. laevis* testis (Reid 1980; Bartsch & Rohr 1980). The model utilises morphological structures such as seminiferous tubules and interstitial components, and provides quantitative values of relative fractional volumes ( $V_v$ ) and percentages of the principal tissues and cells. This methodology generates quantitative data that can be applied in risk assessment of toxicological effects for histological endpoints in toxicological studies both in the field and laboratory.

*Xenopus laevis* (African Clawed Frog) is a truly aquatic frog that occurs naturally in water bodies throughout South Africa. This species is found in most bodies of water including ponds in the maize-production areas (Du Preez 1996). Maize is grown intensively in the northwestern and central parts of South Africa. This region experiences dry winters and has somewhat

unreliable summer rains. Due to surface runoff, these ponds often contain residues of atrazine, a herbicide, and various pesticides used in maize production (Hecker *et al.* 2004). Maize is usually planted in October and November and is treated with atrazine and related triazines in November-December. Runoff occurs primarily during the South African spring and summer rains. To ascertain whether exposure to atrazine and pesticides is having adverse effects on *X. laevis* under field conditions, this study tests whether morphological changes were present in the gonads and whether there were effects on laryngeal size of *X. laevis* collected from MGA and NMGA of South Africa.

## MATERIALS & METHODS

*Collection Sites.*—A detailed description of the collection sites are given elsewhere (Hecker *et al.* 2004). Briefly, sites in the MGA were selected based on the presence of *X. laevis*, proximity to maize production, previous and planned use of atrazine and terbuthylazine, and presence of atrazine in samples taken during the winter before the study commenced. The sites in the NMGA were selected based on the presence of *X. laevis*, lack of maize production and the concentration of atrazine and terbuthylazine at background concentrations in the water during winter. MGA sites were situated in the Viljoenskroon area (Free State Province, South Africa) and NMGA sites 50 km to the north near the city of Potchefstroom (North West Province, South Africa).

*Collection and tissue sampling of X. laevis.*—During the end of the rainy season (April through May 2002) male and female frogs ranging from 8.0 g to 112 g were collected using bucket traps, transferred to labelled containers and transported to the laboratory at the Potchefstroom University for processing. Frogs were kept individually in dechlorinated tap water in 2-l plastic containers for 48 h to recov-

er from capture stress. Animals were killed by immersion in tricaine methanesulfonate (0.5 g/L, MS-222), weighed and the snout-vent length (SVL) measured. Gonads and larynxes were dissected and weighed. The left gonad was preserved in Bouin's fixative for histological examination. The contralateral gonad was used to assess aromatase activity in a separate study by Hecker *et al.* (2004). The larynx was removed, weighed, and fixed in Bouin's fixative. The laryngeal-somatic index (LSI) was calculated using the following formula:

$$\text{LSI (\%)} = (\text{larynx mass} / \text{body mass}) \times 100.$$

**Histological Evaluations.**—Preserved testicular tissue samples were dehydrated in ethanol of ascending concentrations, embedded in paraffin, serially sectioned at 5  $\mu\text{m}$ , and stained with hematoxylin and eosin. Three sections were selected from the rostral, middle, and caudal regions of each testis. Selection of sections was based on the presence of clearly identifiable seminiferous tubules and spermatogenic cells. Three non-overlapping areas were photographed at 60X magnification from each section from the rostral, middle and caudal regions of each testis using an Olympus P12 digital camera attached to an Olympus BX-15 microscope. 1). The photomicrographs were subjected to histometric analyses to determine the relative fractional volume of each component and the stages of spermatogenesis. Each digital image was loaded into a PowerPoint® file with 35 x 1 square-inch grid overlay. The photomicrograph with the grid overlay was printed, and the cell or tissue type under each crossbar was identified and counted. Three different persons independently assessed each photomicrograph. The average of the three counts was used to determine the representative fractional volume of spermatogonia, spermatocytes, spermatozoa, and blood vessels, as well as connective tissue and other cell types. Every twentieth section was screened for testicular oocytes. Testicular oocytes were characterised by the presence of

basophilic cytoplasmic inclusions (yolk platelets) and clear follicular cell layers and nucleoli, as described by Dumont *et al.* (1972).

**Statistical Analyses.**—Because collection sites were considered the basic units of observation (and differences among sites in the same region were expected), the site means were used in most statistical tests. Body mass data were analysed using Student's unpaired *t*-test and Mann-Whitney U-test. The LSI-data were analysed with a Student's unpaired *t*-test. The histopathology data were subjected to a two-sample *t*-test (assuming equal variances) to test for differences between the NMGA and MGA sites. The criterion for significance in all statistical tests was  $P < 0.05$ . The data were analysed using SAS statistical software (SAS 1982).

## RESULTS

**Body Mass and LSI.**—The body mass and LSI for females and males from the NMGA and MGA sites are presented in Table 1. Based on Student's unpaired *t*-test (NMGA,  $P = 0.0001$ ) or Mann-Whitney U-test (MGA,  $P = 0.0003$ ), the body mass of females was significantly greater than that of males from their respective sites. In addition, the combined laryngeal cartilage mass for females was significantly greater than those of males based on Mann-Whitney U-test for both sites ( $P = 0.0167$  for NMGA,  $P = 0.0946$  for MGA). Male LSI was significantly greater than those of females from respective sites based on Student's unpaired *t*-test (NMGA and MGA,  $P < 0.0001$ ).

**Gross Morphology.**—The mean SVL (Table 2) of females was greater than in males, with the largest female specimens collected at site MGA-6. The mean of the mean SVL of female frogs at NMGA sites (NMGA-1, NMGA-3, and NMGA-6) was 71.8 mm, and did not differ (two-sided two-sample *t*-test assuming equal variance  $P = 0.75$ ) from the mean of the mean

Table 1. Body and laryngeal mass and laryngeal somatic indices (LSI) in adult *Xenopus laevis* collected from maize-growing areas and non-maize-growing areas of South Africa.

Site	N	Sex	Body mass (g)	Laryngeal Mass (g) <sup>a</sup>	Laryngeal Somatic Index (x 100)
NMGR	55	♂	26.1 ± 1.78	0.51 ± 0.03	2.03 ± 0.05***
	60	♀	36.5 ± 1.91*	0.63 ± 0.04**	1.72 ± 0.04
MGR	44	♂	27.6 ± 1.61	0.54 ± 0.03	2.00 ± 0.04***
	48	♀	43.0 ± 3.31*	0.71 ± 0.06**	1.60 ± 0.05

<sup>a</sup>Combined mass of laryngeal cartilage and muscle.

\*Significantly greater than males from respective site based on Student's unpaired t-test (NMGR,  $P = 0.0001$ ) or Mann-Whitney U-test (MGR,  $P = 0.0003$ ).

\*\*Significantly greater than male based on Mann-Whitney test for both sites ( $P = 0.0167$  for NMGR,  $P = 0.0946$  for MGR).

\*\*\*Significantly greater than female from respective site based on Student's unpaired t-test (NMGR and MGR,  $P < 0.0001$ ).

SVL at MGA sites (MGA-1, MGA-3, MGA-4, MGA-6, and MGA-8), which was 73.9 mm.

There were no significant differences in the mean mass (36.5 g) of female frogs at NMGA sites and the mean mass (42.8 g) of female frogs at MGA sites (two-sided two-sample  $t$ -test assuming equal variances;  $P = 0.53$ ). The mean mass of male frogs at NMGA sites was 25.8 g, and this did not differ from the mean mass (27.0 g) of male frogs at MGA sites (two-sided two-sample  $t$ -test assuming equal variances;  $P = 0.24$ ). The mean mass of the left testis in male frogs from MGA sites was 18.6 mg, and the mean mass of the left testis in male frogs at NMGA sites was 23.3 mg. These did not differ significantly as determined by a two-sided two-sample  $t$ -test, assuming equal variances, ( $P = 0.29$ ). The GSI data for these animals have been reported in a separate manuscript (Hecker *et al.* 2004). In general, the mean GSI for *X. laevis* males collected in NMGA sites was less than those of *X. laevis* from the MGA. The GSI of female *X. laevis* varied more than for males.

*Gross Morphological Assessment of Gonadal Anomalies.*—A relatively small incidence of testicular abnormalities, such as underdeveloped or missing testes, was observed (Fig. 1a, 1b) in males. No abnormal ovaries were

observed in females. Of the 101 male frogs examined, two had discontinuous testes and four had testes of unequal size. These abnormalities were found in both the NMGA and MGA.

*Testicular Histometric Analysis.*—The assessment of the distribution of testicular cell types in male *X. laevis* revealed that the mean of the among-individual means of the histometric measurements for the three NMGA sites was not statistically different from the mean of the among-individual means for the five MGA sites (two-sample  $t$ -test assuming equal variances) for any of the cell types (Table 3). All animals examined histologically exhibited normal-appearing testes with visible spermatozoa.

*Assessment of Testicular Oocytes.*—A small incidence of testicular oocytes (Figs. 2 and 3) was observed in both regions (NMGA = 3%; MGA = 2%). Based on the Dumont (1972) classification scale, these testicular oocytes were stage I pre-vitellogenic oocytes that measured less than 300  $\mu\text{m}$  in diameter. A follicular cell layer containing basophilic inclusions surrounded each.

Table 2. Mean ( $\pm$  SEM) body mass, snout-vent length (SVL) and gross gonadal abnormalities in adult *Xenopus laevis* collected from non-maize-growing areas (NMGA) and maize-growing areas (MGA) of South Africa.

	NMGA-1	NMGA-3	NMGA-6	MGA-1	MGA-3	MGA-4	MGA-6	MGA-8
♂ mass (g)	19.4 $\pm$ 5.1 N = 18	20.7 $\pm$ 13.6 N = 17	37.3 $\pm$ 12.5 N = 20	23.4 $\pm$ 16.5 N = 8	32 $\pm$ 8.9 N = 10	29.9 $\pm$ 4.7 N = 10	26 $\pm$ 13.8 N = 10	23.09 $\pm$ 9.7 N = 6
♀ mass (g)	32.8 $\pm$ 11 N = 20	35.3 $\pm$ 20 N = 20	41.2 $\pm$ 10 N = 20	29.1 $\pm$ 19 N = 6	39.4 $\pm$ 8 N = 10	48.8 $\pm$ 19 N = 10	66.9 $\pm$ 23 N = 10	29.8 $\pm$ 19 N = 12
♂ SVL (mm)	58 $\pm$ 6.6 N = 18	56.9 $\pm$ 10.9 N = 17	72.1 $\pm$ 7.2 N = 20	59.2 $\pm$ 9.5 N = 8	66.6 $\pm$ 7 N = 10	63.8 $\pm$ 3.4 N = 10	62.7 $\pm$ 9.6 N = 10	60.7 $\pm$ 7.3 N = 6
♀ SVL (mm)	69.5 $\pm$ 8 N = 20	68.9 $\pm$ 14 N = 20	76.9 $\pm$ 7 N = 20	64.5 $\pm$ 14 N = 6	74.4 $\pm$ 7 N = 10	77.5 $\pm$ 11 N = 10	88.5 $\pm$ 10 N = 10	64.5 $\pm$ 14 N = 12
# gross ( $\sigma/\rho$ ) Abnormalities	0/0	0/0	3/0	0/0	2/0	0/0	0/0	0/0

## DISCUSSION

This study, using stereological analysis of the fractional volume of testicular cell types, demonstrated no significant differences in mature *X. laevis* from NMGA and MGA of South Africa. This suggests that spermatogenic cell cycles and the process of sperm maturation are similar in NMGA and triazine-exposed areas. Data from the present study also supported the conclusion of a continuously active spermatogenic cycle in *X. laevis* (Kalt 1977). Previous studies have used tritiated thymidine autoradiography to determine the duration of meiotic prophase and spermiogenesis in *X. laevis* (Kalt 1976). Wide variations for counts between testes from single animals and among testes from different animals, whether sampled at the same time or at different times of the

year, have been reported (Bartsch *et al.* 1978) and are consistent with the concept of a continuously-active spermatogenic cycle. This possibly explains some of the variations we observed within and among sites in both the NMGA and MGA. It should be noted that this is not case for other frogs from areas with short intense breeding seasons. However, *X. laevis* has an extended breeding season and breeds opportunistically in response to environmental conditions.

The observation of a small incidence of testicular oocytes in frogs from both the NMGA and MGA sites raises several basic biological questions relative to the development and differentiation of *X. laevis* gonads. For example, is the presence of testicular oocytes a natural phenomenon in the course of gonadal ontogeny of these animals? Is the presence of these oocytes

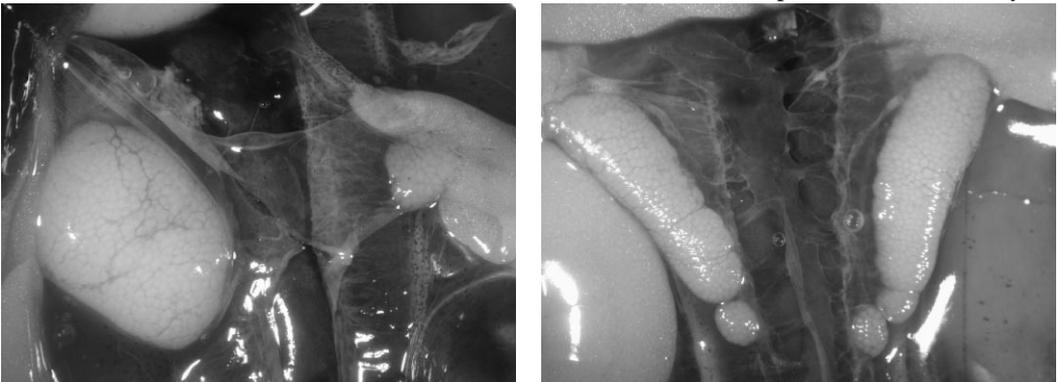


Figure 1. Examples of testicular deformities. Left photo shows an underdeveloped left testis, and right photo shows a pair of discontinuous testes.

Table 3. Histometric evaluation of testicular cell types in *Xenopus laevis* collected from non-maize-growing area (NMGA) and maize-growing area (MGA) in South Africa. Values represent mean percent volume ( $V_v$ ) of total based on the stereological analyses of 55 NMGA and 44 MGA samples.

Site		Spermatagonia	Spermatocytes	Sperm	Connective Tissue and Other Cells	Blood Vessels
	$N$	$V_v$	$V_v$	$V_v$	$V_v$	$V_v$
NMGA 1	18	3.0	25.6	55.9	14.9	0.5
NMGA 3	17	4.5	34.3	46.3	14.6	0.3
NMGA 6	20	2.8	22.6	60.3	13.9	0.3
<b>Mean of NMGA</b>		<b>3.5</b>	<b>27.5</b>	<b>54.2</b>	<b>14.5</b>	<b>0.4</b>
MGA 1	8	3.4	28.8	53.4	13.7	0.8
MGA 3	10	3.6	24.9	58.1	13.2	0.2
MGA 4	10	3.0	24.5	59.8	12.6	0.2
MGA 6	10	3.2	20.8	62.4	13.2	0.3
MGA 8	6	2.9	30.1	52.2	14.5	0.2
<b>Mean of MGA</b>		<b>3.2</b>	<b>25.8</b>	<b>57.2</b>	<b>13.5</b>	<b>0.3</b>
<i>P</i> -values in <i>t</i> -tests		0.62	0.64	0.48	0.075	0.73

independent of any influence mediated through exposure to atrazine or other pesticides used in the production of maize? It should be noted that vitellogenic follicles were not observed. It is clear that the basic development of the testis in *X. laevis* needs to be examined chronologically, from anlagen to adult, to determine the frequency of incidence of these oocytes.

Recent data for *X. laevis* from these sites (NMGA and MGA) indicated a significant negative correlation between waterborne concentration of atrazine and plasma concentration of estradiol (Hecker *et al.* 2004). However, the investigators found no significant relationship between aromatase activity and exposure to

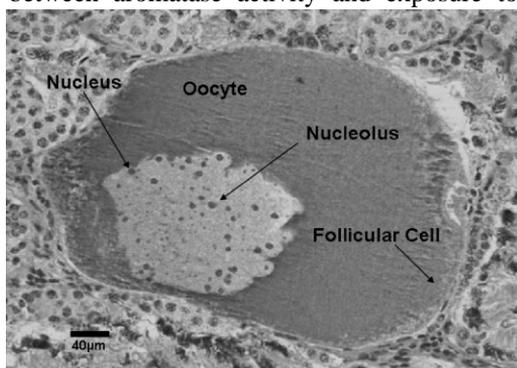


Figure 2: Photomicrograph illustrating a testicular oocyte from *Xenopus laevis*. Haematoxylin and eosin.

atrazine (Hecker *et al.* 2004). Data reported by Coady *et al.* (2004) indicated that there was no dose-response relationship between atrazine and incidence of testicular oocytes following laboratory exposure. Therefore, it appears that the presence of testicular oocytes is not related to the effects of atrazine or other chemicals presents in MGA or NMGA. Thus, although the presence of testicular oocytes has been postulated to be caused by exposure to atrazine, the results of our studies offer little support for this hypothesis.

Laryngeal development in frogs is a sexually dimorphic process, and the formation of a larynx capable of male calling behaviour is andro-

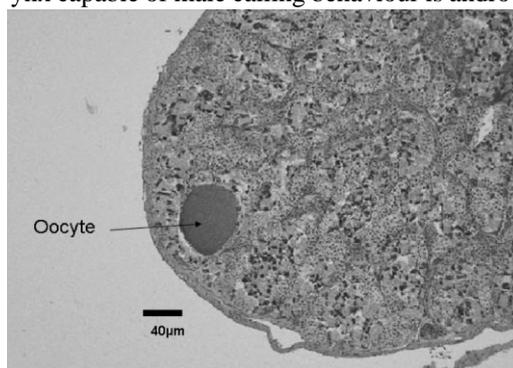


Figure 3: Photomicrograph showing a testis with normal stages of spermatogenesis and a testicular oocyte. Haematoxylin and eosin.

gen-dependent. Under normal conditions the laryngeal dilator muscle of male *X. laevis* is larger than that of females (Sassoon & Kelley 1986; Tobias *et al.* 1993). It has been hypothesised that atrazine could act as an endocrine disruptor by decreasing plasma concentrations of testosterone in *X. laevis* resulting in a decrease in laryngeal dilator muscle volume (Hayes *et al.* 2002a). Thus, dilator muscle size was selected as an indicator of disruption of plasma hormone balance, especially of androgens during development. Theoretically, this measurement endpoint can serve as an integrating measure of androgen-dependent processes that would respond to subtle changes in androgen status during critical periods of development when it is not possible to measure plasma hormone concentrations. Such a measure should be able to detect changes that occur in small windows during development as well as changes in androgen status in tissues that might not be observed in the plasma.

Our observation that there was no difference in laryngeal dilator muscle size in either males or females from the MGA and the NMGA is consistent with a laboratory study that observed no effect on laryngeal dilator muscle size (Carr *et al.* 2003) in atrazine-exposed frogs and also fails to support the observation of (Hayes *et al.* 2002b) in which exposure to concentrations of atrazine at concentrations as low as 0.1 ug/L caused decreased larynx muscle size in atrazine-exposed *X. laevis*. However, in that study, there was no dose-dependent response as would be expected. In our study, male *X. laevis* had greater laryngeal muscle size than did females.

Based on population data reported for *X. laevis* from the MGA and NMGA sites in southern Africa (Hecker *et al.* 2004), it seems unlikely that low incidence of testicular oocytes resulted in functional sterility or reduces fecundity. The spermatogenic tissue data further support the conclusion that frogs from the two different

regions present similar volumes of spermatogenic cells that are not significantly different. Further evaluation is necessary to determine if sporadic testicular oocytes would have an impact on reproduction at the population-level.

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