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Reproductive Biology of the African Clawed Frog, *Xenopus laevis*
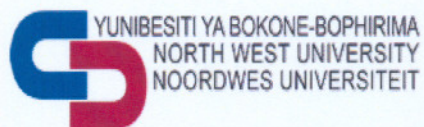
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Dissertation submitted in partial fulfilment of the requirements for the degree Magister in Environmental Sciences at the North-West University (Potchefstroom Campus)

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Potchefstroom

YUNIBESITI YA BOKONE-BOPHIRIMA
NORTH WEST UNIVERSITY
NOORDWES UNIVERSITEIT

Potchefstroomkampus

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For My Parents

Giel and Marie

TABLE OF CONTENTS

Acknowledgements

List of Figures

List of Tables

Summary / Opsomming

Chapter 1: Introduction and Literature Overview

Chapter 2: Study Area, Materials and Method

Chapter 3: Results: Seasonal Fluctuation in the Reproductive Cycle of *Xenopus laevis*

ACKNOWLEDGEMENTS

LIST OF FIGURES

LIST OF TABLES

SUMMARY

Apart from the mouse, rat, and chicken, the clawed frog, *Xenopus laevis*, is probably the best-studied chordate laboratory animal. Although this animal has been studied for decades around the world we still know relatively little about its biology, including its reproduction under natural conditions. It is surprising that we know so little about an animal for which the entire genome has been sequenced. The aim of this study was to characterise the reproductive biology of the clawed frog over a period of a year. On a monthly basis, 10 males and 10 females were collected from each of three study sites. Morphometric measurements were taken for all animals. Blood samples were taken, gonads examined at gross morphological level and gonads fixed for histological analysis. Gross morphological anomalies showed prevalence between 2.1% and 3.8% at the three study sites. Gonads were serially sectioned and the reproductive state of the gonads determined by means of histometric analysis as a function of seasonal changes. Photomicrographs were taken of the gonads under a microscope and the cell types were scored quantitively. The histological sections of the gonads were examined for gonadal anomalies, including testicular oocytes. Testicular oocytes were present at all three study sites with prevalence between 12.5% and 20.2%. Water quality parameters and environmental data were collected at all three sites for the duration of the study. External sex characters of *Xenopus laevis* were also classified and each individually scored. The age structure of *Xenopus laevis* populations was also determined at the three study areas. Hormonal analysis was also done to determine the concentrations of sex steroids testosterone and estradiol. The ecological aspects of *Xenopus laevis* reproduction were also characterise at a fourth study site. Rainfall had the determining effect of *Xenopus laevis* reproduction. It was also found that the clawed frog had an extended breeding season from August to March.

OPSOMMING

Naas die muis, rot en kuiken, is die gewone platanna, *Xenopus laevis*, waarskynlik die mees bestudeerde gewerwelde laboratoriumdier ter wêreld. Hoewel die platanna al vir dekades regdeur die wêreld bestudeer word, is daar betreklik min bekend omtrent die basiese biologie, insluitende die basiese voortplantingsbiologie onder natuurlike omstandighede. Dit is veral vreemd as ons in ag neem dat die platanna se volledige genoom al beskryf is. Die doel van hierdie studie was om die voortplantingsbiologie van *Xenopus laevis* vir een periode van 'n jaar te bestudeer. Op 'n maandelikse basis is 10 mannetjies en 10 wyfies uit drie natuurlike damme in die Potchefstroom omgewing versamel. Morfometriese afmetings is van elke individu geneem. Bloed is geneem, waarna die gonades uitwendig bestudeer en gefikseer is vir histologiese ontleding. Tussen 2.1% en 3.8% het uitwendige morfologiese afwykings getoon. Na histologiese seriesneë is histometriese analise gebruik om die toestand van die gonades as 'n funksie van seisoenale verandering te bepaal. Fotomikrogramme is van die gonads geneem en die verskillende seltipes is gekwantifiseer. Snitte deur die gonades is ook bestudeer vir testikulêre abnormaliteite, insluitende testikulêre oösiete. Testikulêre oösiete is by al drie die studie-areas gevind met 'n voorkoms tussen 12.5% en 20.2%. Waterkwaliteitsparameters is by al die damme geneem. Eksterne geslagskenmerke is oor die duur van die studie gemoniteer. Ouderdomprofiele van *Xenopus laevis* by al drie damme is bepaal. Hormoonanalises vir testosteroon- en estradiolkonsentrasies is in die VSA bepaal. Gevallestudie was by 'n vierde studie-area gedoen om die ekologiese aspekte van *Xenopus laevis*-voortplanting te bepaal. Daar is gevind dat reënval die bepalende faktor is vir *Xenopus laevis* voortplanting en dat 'n verlengde broeiseisoen vanaf Augustus tot Maart voorkom.

CHAPTER 1

INTRODUCTION AND LITERATURE OVERVIEW

"Investigations into basic physiology must be given a higher priority if we are to understand and prevent adverse effects of ecotoxins in the environment."

(Palmer, 2000)

This quote by Palmer not only applies to the physiology, but also to the biology of the test animals in general. *Xenopus* has been exploited over decades and apart from the mouse, the rat and the chicken it is probably the best-studied laboratory animal today. For this reason is it surprising that the reproductive biology of *Xenopus* is not thoroughly studied under natural conditions.

1.1 Introduction to *Xenopus laevis*

The generic name *Xenopus* is derived from the Greek words "xenos" meaning strange or unusual, and "pous" which means foot. The specific name *laevis* means smooth and relates to the slimy surface of the frog (Brown, 1970 and Du Preez, 1996). *Xenopus laevis* is smooth and streamlined, with large, webbed feet and sensory lateral-line organs (Figure 1.1). The head is small and flattened with large eyes on top. Sensory organs are arranged around the eyes and along the side. *Xenopus* is adapted for life in water with strong legs, webbed feet and clawed toes (Channing, 2001). *X. laevis* is a non-tropical species covering most of southern Africa from the Cape northwards to Angola, and to Lake Rudolf (Kenya) in the east, and from there westwards and towards the north to Cameroon and Nigeria (Brown, 1970), excluding the Zaire Basin and the hotter lowlands of eastern Africa (Tinsley *et al.*, 1996). The distribution range can be described in short as the sub-Saharan African savanna (Channing, 2001) (Figure 1.2). The clawed frog is ubiquitous on the highveld and is found in practically every type of water-body south of the Sahara (Kobel *et al.*, 1996).

Figure 1.1: Photograph of *Xenopus laevis*, showing the position of the claws and sensory lateral-line organs.

Figure 1.2: Distribution map of *Xenopus laevis* in Africa.

Xenopus is not averse to polluted water, but is most abundant in waterholes and dams, both large and small (Balinsky, 1969). Individuals appear to occupy whatever aquatic habitat is available with no evidence of preference for specific biotypes within a geographical range. *X. laevis* is most common in stagnant and sluggish ponds with a wide variation in the water chemistry (Tinsley *et al.*, 1996).

With the narrowing of the mouth, the fingers became important aids in feeding. No other amphibian stuffs food into the mouth with their fingers or even holds the food with their forelimbs while devouring it. The tongue-less *Xenopus* is very adapted in handling its prey with its fingers and forcing it into the comparatively small mouth (Noble, 1954). *Xenopus* feed and breed under water and that makes them the only South African amphibian that occupies a completely aquatic habitat. They can swim extremely fast either backwards or forwards when disturbed and can stay under water for prolonged periods without coming to the surface for air.

If the pools should dry up, *Xenopus* adults will bury themselves in the mud and aestivate until the next rain (Rose, 1950 and Tinsley *et al.*, 1996). Hewitt and Power (1913) reported that *X. laevis* remained in good condition for eight months during aestivation. *Xenopus* shows a remarkable ability to tolerate starvation. Merkle and Hanke (1988) monitored *X. laevis* for 12 months without food during laboratory experiments. During the first 4 to 6 months, stored carbohydrates and lipids are used. After this, protein is catabolised from muscle and body weight drops. The mechanism of survival includes a switch from the excretion of toxic ammonia to the production of urea, which accumulates in the blood, liver and muscle during dehydration (Balinsky *et al.*, 1961). When entering water, very large quantities of urea are excreted. Wager (1986) states that *X. laevis* has the ability to breathe through its skin, which is well supplied with blood. Even the interdigital webbing has numerous blood vessels. The species can also slide overland with powerful thrusts of the hind limbs, but only under conditions where their skin can be kept damp by rain or dew (Brown, 1970; Loveridge, 1953 and Hewitt & Power, 1913).

Both male and female *Xenopus* call with soft vibrating trills emitted underwater that are almost inaudible to the human ear (Loumont, 1981 and Yager, 1992). They call while floating a few centimetres below the surface with hands held out in a snatching posture. Kelly (1980) and Emerson (2001) state that calling in males is under the control of androgens and that male advertisement calls in frogs are one of the most energetically expensive activities of amphibians. Obert (1977) also argues that upon hearing species-

specific vocalisation, androgen levels in breeding males are increased. Breeding takes place in pools and dams, but it is doubtful whether the frog can breed in more rapid streams with stony beds, as the eggs are dispersed on submerged vegetation and the tadpoles are planktonic (Balinsky, 1969). The breeding season extends from the beginning of September to the middle of March. The breeding season of *Xenopus,* which extends over six months, is the longest of all spring-and-summer breeding species. *Xenopus* will breed more than once when conditions are favourable but seldom more than twice in a year (Wood, 1965 and Tinsley *et al.,* 1996). Spawning usually occurs during the night (Balinsky, 1969).

Climatological conditions and, in particular rainfall and temperature, determine the geographic and ecological distributions of amphibians, timing, and intensity of feeding, reproduction, and migration. Breeding often takes place in a specific season and field observations suggest that climatological conditions on the days when spawning takes place or during preceding days play an important part in triggering the process of reproduction. With *X. laevis* being a fully aquatic frog, one would expect that climatological conditions would have less of an effect on *X. laevis* but this is not the case (Heyer *et al.,* 1994). *X. laevis* can tolerate quite a wide range of temperatures from 10°C to 28°C, the optimum being 23°C (Brown, 1970; McCoid & Fritts, 1980 and Moron, 1947). Berk (1938) and Savage (1971) reported that, when the mid-afternoon temperature at the surface of the water rises above 21°C, spawning would be abundant on the following day. Rainfall strongly influences amphibian activity, distribution and dispersion patterns, reproductive cycles, rates of growth and development (Heyer, 1994). The availability of food may also act as a secondary stimulus for breeding in *X. laevis.* Under natural conditions, the effect of heavy rain would be to wash fresh sediments into ponds, enriching the nutrient status of the water (Tinsley *et al.,* 1996).

Instinctive habits, often quite different in the two sexes, appear during the breeding season. These are under the influence of steroid hormones secreted by the gonads (Wilson, George & Griffin, 1981 and Evans, 1988) and may be classified as secondary sexual characters (Noble, 1954). Females show the presence of three labia, two dorsal

and one ventral to the cloaca (Figure 1.3). They become swollen and more prominent in the breeding season and show redness just before spawning. This could be used to determine the reproductive state of the female. In males, the presence of nuptial pads during the breeding season will indicate the reproductive state of the male (Brown, 1970). Nuptial pads are black excrescences on the inner ventral surface of the forelimb of the males (Figure 1.4) and the function thereof is to secure a firm grip on to the female during amplexus.

Figure 1.3: Photograph illustrating the posterior halves of a male (left) and a female (right) *Xenopus*. Note the swollen cloaca of the female. The red colour of the swollen cloaca indicates that the female is about to spawn or has just spawned.
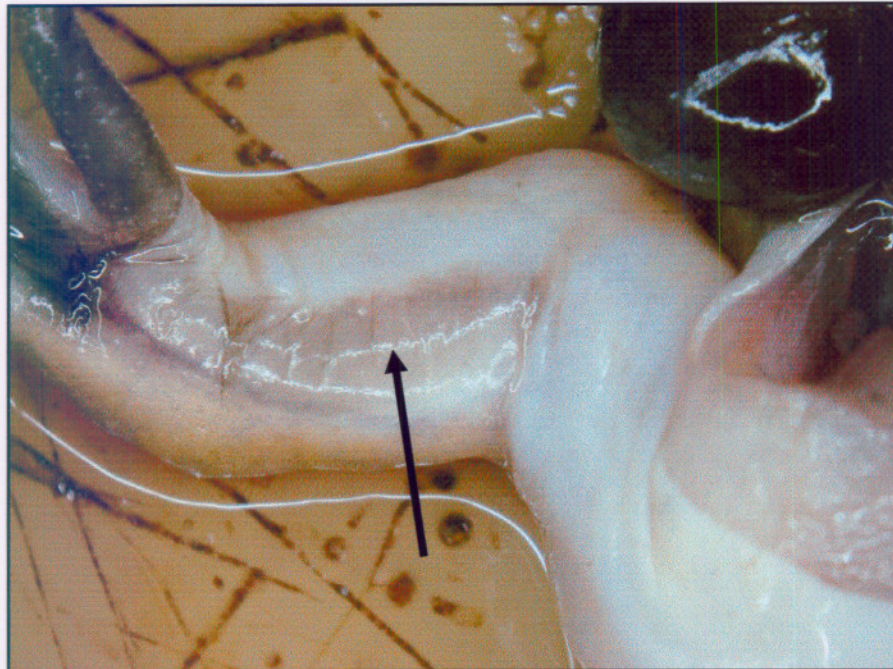
Figure 1.4: Photograph of the ventral surface of the forearm of the male *Xenopus* showing the dark coloured nuptial pads.

The gonads, while primarily organs of reproduction, release hormones into the blood which have an important function in stimulating the growth and maintaining the development of the secondary sexual characters, for example, the nuptial pads. The secondary sexual characters include differences in red cell count, lung size, behaviour patterns, and many other structural and physiological differences between the sexes. It seems that the testis induces and maintains the secondary sexual characters of the male. The stomal cells surrounding the lobules of the testis produce the testicular hormones of amphibians (Noble, 1954 and Nishimura, 1997).

1.2   Early *Xenopus* Research

Research on *Xenopus* during the 1900s was characterised by a slow start and then an explosion of papers when the importance of *Xenopus* as a laboratory animal was noticed (Zwarenstein & Burgers, 1955). This was reflected in the fact that 260 papers were published on *X. laevis* from 1920 to 1945 (25 years) and then almost the same

number of publications during the following 7 years from 1946 to 1953. The interest was sparked by the discovery by Shapiro and Zwartstein (1933) that *Xenopus* could serve as a pregnancy assay for humans. When urine from a pregnant woman is injected subcutaneous into a female *Xenopus* she will spawn that night (Barton, 1953; Cowie, 1948; Elkan, 1946; Polack, 1946 and Rasmussen, 1946). Soon this assay became common practice in various countries which caused a huge demand for *Xenopus* females. This led to the annual export of thousands of *X. laevis* all over the world.

During the late 1940s, the interest in *Xenopus* had almost completely shifted from morphological studies to experimental physiology. Authors such as Hey (1946), Keiper (1949) and Schwabacher (1953) published papers on the breeding and husbandry of *X. laevis* in captivity. This contributed to a large extent to the use of *Xenopus* as a laboratory animal. Gurdon (1996) list a few reasons why *X. laevis* became increasingly more popular as a laboratory animal:

- *Xenopus* can be induced to mate and provide fertile embryos by the gonadotrophic hormones of other vertebrate species.
- Permanent aquatic lifestyle that allowed people to keep them in water tanks that are cleaned more easily.
- *Xenopus* is resistant to disease and infection.
- A fertilised egg can be grown to a sexually reproductive adult in one year.
- *Xenopus* produces large-sized embryos and cells for molecular studies and messenger RNA is very efficiently translated when microinjected into oocytes.
- *Xenopus* is a reliable source of high quality fertile eggs and oocytes.

In the early days, studies on *Xenopus* focused on embryology (Balinsky, 1951), development (Bruce, 1950; Fox, 1950; Millard, 1949 and Peterson, 1949), and metamorphosis (Cordier, 1949; Newth, 1948 and Toivonen, 1952). Limited work has also been conducted on the role and function of the thyroid (Dodd & Landgrebe, 1953 and Parkes, 1946) and the gonads, in particular the phenomenon of sex reversal (Chang, 1953) and gonad transplantation (Chang, 1954). Endocrinology research in general was also slow to start (Robbins, 1949), but gonadotropin was studied in more

detail. Robbins, Parker & Hobson (1947) described the effects of gonadotropins on the male *X. laevis* and Thorborg (1950) and Landgrepe (1948) used *X. laevis* as a test animal for biological assays of gonadotropin. Hobson (1952) published papers on the conditions for the release of spermatozoa in male *X. laevis* in response to chorionic gonadotropin.

During the 1960s, the importance of amphibians in biological investigations was noted (Moore, 1964). More papers were published on amphibian metabolism (Brown, 1964; Cantarow & Schepartz,1962; Deuchar, 1956; Yamamoto, 1960 and Silver & Balinsky, 1961), blood and respiration (Foxon, 1964; Czopek, 1955 and Ewer, 1959), physiology of the amphibian heart (Adrian, 1960; Brady, 1964 and Thomas, 1960), endocrinology of amphibia (Gorbman, 1964; Burgers & Boschman, 1953 and Leaf, 1960), developmental physiology (Barth, 1964 and Weber, 1954) and regeneration (Rose, 1964 and Tschumi, 1957). Moore (1964) made the statement that, considering all the publications, there are still many and large gaps in the knowledge of amphibian physiology.

What is the situation today on *Xenopus* research? *Xenopus* has been employed intensively in laboratory-based research for over 50 years in fields such as physiology, biochemistry, endocrinology, and developmental biology. All this emerged against an almost total lack of information on ecology and species diversity (Tinsley & Kobel, 1996). Research work is focusing on cell and molecular biology and systematics and genetics of *Xenopus* (Cannatella & Trueb, 1988; De Sà & Hillis, 1990; Giorgi & Fischberg, 1982; Graf, 1989; Kobel & Du Pasquier, 1986; Müller, 1977; Robert *et al.*, 1990 and Schmid & Steinlein, 1991). There is an endless field of study opportunities when looking at only one aspect, for example reproductive biology. There are gonadal development (Iwasawa & Yamaguchi, 1984 and Witchi, 1971), chromosomal determination of gonadal sex (Denny *et al.*, 1992; Lovell-Badge, 1993; Griffiths, 1991 and Harley *et al.*, 1992), hormones (Wibbels & Crews, 1992; Dorizzi *et al.*, 1991 and Tobias *et al.*, 1991), steroid secretion (Witschi, 1971; May & Knowland, 1980; Kawahara & Kohara, 1987; Baulieu *et al.*, 1978; Kelley & Dennison, 1990; and Smith, 1989),

secondary sexual differentiation (Witschi, 1971; Hannigan & Kelley, 1986 and Watson & Kelley, 1992) and reproductive behaviour (Hannigan & Kelley, 1986; Kelley, 1982; Lambdin & Kelley, 1986 and Wetzel & Kelley, 1983). Not even considered in this are the many opportunities for ecotoxicological research. In the early years of research on *Xenopus*, little attention was paid to the effect that toxic substances have on amphibians and reptiles (Sparling *et al.*, 2000). Metal residue, acidification and non-chlorinated pesticides were the focus of most research on amphibians. Limited work was conducted on the effects of oils, dioxins, furans and DDT. Sparling (2000) noted that the ecological importance of amphibians did not play a role in ecotoxicological research, but only anthropocentrical factors, such as economical value of certain wildlife species.

Atrazine and its alleged effect on amphibians is one such an example. It was introduced as an herbicide in 1957 for the control of broadleaf and grass weeds in corn and other crops (Du Preez *et al.*, 2005 and Giddings *et al.*, 2005). Atrazine can reach surface water systems through run-off, seepage, and aerial drift during application. Herbicides are relatively persistent in freshwater (Solomon *et al.*, 1996). Several field surveys have shown that amphibian deformities may be associated with exposure to pesticides and herbicides (Ouellet *et al.*, 1997 and Sower *et al.*, 2000). Smith (2005) asked the following questions: Are the anomalies that occur in amphibians normal or not? Are the occurrences of these anomalies due to the effects of herbicides or pesticides? These questions still need to be answered. There are still many research opportunities for species that are considered to be of low economical value. Species of low economical value will first become important when one considers the ecological importance of the conservation of biodiversity.

1.3   History of the Study and Justification

The present study formed part of a much larger phase-orientated project, funded by Syngenta Crop Protection Incorporated, USA. The main objective of the larger study was to determine whether the broadleaf herbicide atrazine has any adverse effects on *X. laevis* in its natural habitat. The project was divided into 5 phases, namely:

Phase A - Evaluate sites and compare populations.

In this study five exposed and three reference sites were evaluated in terms of population size, size of frogs, sex ratio and age profile. Exposed sites and reference sites were found to be very similar in all aspects and no evidence was found that exposed sites show anomalies at the population level (Du Preez *et al.*, 2004)

Phase B - Monitor pesticide concentrations at sites over one field use season.

Water and sediment samples were taken on a regular basis and analysed for pesticides. (Du Preez *et al.*, 2005).

Phase C - Hormonal and histological studies,

At the end of the season monitored for pesticides representative *Xenopus* samples were collected at each site and dissected. One gonad per specimen was histologically examined (Smith *et al.*, 2005) while the remaining gonad and a blood sample were analyzed for reproductive hormones and aromatase (Hecker *et al.*, 2004 and 2005).

Phase D - Microcosm study

In order to study the effects of atrazine alone on clawed frogs a microcosm study was undertaken. No intersex specimens were observed. At the histological level, testicular oocytes were observed at all concentrations but appear to be a natural phenomenon and not related to atrazine exposure (Jooste *et al.*, 2005).

Phase E - *Xenopus* reproductive biology (present study).

Difficulties experienced during the interpretation of hormonal levels (Smith, 2003a and Giesy, 2003) stressed the need for a detailed study on the reproductive biology of X. *laevis* in its natural environment. The data on plasma hormones as well as on aromatase activity obtained during this study showed high variability both within and between the populations sampled.

Nevertheless, there appeared to be some relationship between the exposure to atrazine or its metabolite Diaminochlorotriazine (DACT) and plasma T and E2 concentrations in male or female *X. laevis*. It is known that titres of sex steroids change dramatically with the season, age, stage of maturation and also many exhibit diurnal patterns. Time-dependent changes as well as background variability in populations from the wild or in the lab can be greater than chemically-induced alterations, and therefore, it is essential to have detailed background information about these factors. *X. laevis* that were collected during earlier studies (Du Preez *et al.*, 2005) represented a more or less heterogenic population structure with frogs at different maturation stages, and different ages. In order to be able to adequately assess the effects resulting from the exposure to substances that are suspected of interfering with the endocrine system, it is essential to understand both seasonal patterns and natural variability of the investigated parameters under reference conditions.

Xenopus has been employed intensively in laboratory-based research for over 50 years (Gurdon, 1996). From all these studies it became evident that the limited literature on the biology of *Xenopus* in the natural environment was published in old and relatively obscure papers. There are no thorough ecological studies of population biology, interactions within communities, generation times, and growth rates derived from long-termed field-based research. Researchers are so used to the study of *X. laevis* under laboratory conditions, that the conditions under which they live in the wild, are neglected.

Palmer (2000) puts it best when he argues for more studies to be done on the biology of reptiles in order to understand the effects of toxic substances on them. He said that additional research is needed into the broad diversity of anatomical and physiological adaptations of reptilian species. This is also true for amphibian species. The physiology of relatively few species has been studied in detail. Studies of model organisms, such as *X. laevis*, are warranted to understanding basic processes. All too often, interest is not aroused and investigative studies are not begun until a species' numbers are precariously low. Investigations into basic physiology must be given a higher priority if

we are to understand and prevent adverse effects of ecotoxins in the environment. Very little is known about the reproductive physiology and endocrinology of many species. Studies are needed to elucidate the endpoints and mechanisms of actions of endocrine disruptors, such as receptor interactions, alterations of the rate of synthetic and metabolic enzymes, the role of binding proteins, and clearance effects. Clearly, there is a significant amount of research required to enhance our understanding of amphibian ecotoxicology and the underlying physiological processes (Palmer, 2000).

1.4 Study Objectives

The main objectives of this study were to study the reproductive biology of X. laevis with specific reference to:

- Fluctuations in external morphological features in male and female X. laevis under natural conditions.
- Seasonal fluctuations in the reproductive state of the gonads.
- Occurrence of gonadal anomalies, including testicular oocytes.
- Seasonal fluctuations and natural variability in the sex steroid hormones T and E2 in male and female X. laevis.
- Seasonal changes and natural variability in aromatase activity in adult X. laevis of both sexes.
- Population variables and environmental factors that could influence a wild population of X. laevis.

CHAPTER 2

STUDY AREA, MATERIALS AND METHODS

2.1 Selecting the Study Sites

Four sites in the vicinity of Potchefstroom were identified for this study. The sites were selected to comply with the following criteria:

- No application of atrazine in the catchment area of the site.
- Water bodies must be permanent.
- Sites had to be large enough to support a large *Xenopus* population to withstand the destructive sampling.
- Sites should be comparable in nature to limit variation of co-factors.

Two of the sites were used as reference sites in previous studies. Sites A and B were respectively referred to as sites R6 and R1 in previous studies (Du Preez *et al.*, 2004; Du Preez *et al.*, 2005; Jooste *et al.*, 2005 and Smith *et al.*, 2005). Two other sites were identified, evaluated, and found suitable for the study (Figure 2.1). Sampling at sites A and B started in May 2003, at site C sampling started two months later in July of 2003 and at site D in October of the same year. Physical properties of the selected sites are presented in Tables 2.1 to 2.4. To verify the absence of atrazine, water samples were taken at all sites and analysed by Mr. Peet Jansen Van Rensburg at the North-West University, School of Environmental Sciences and Development.
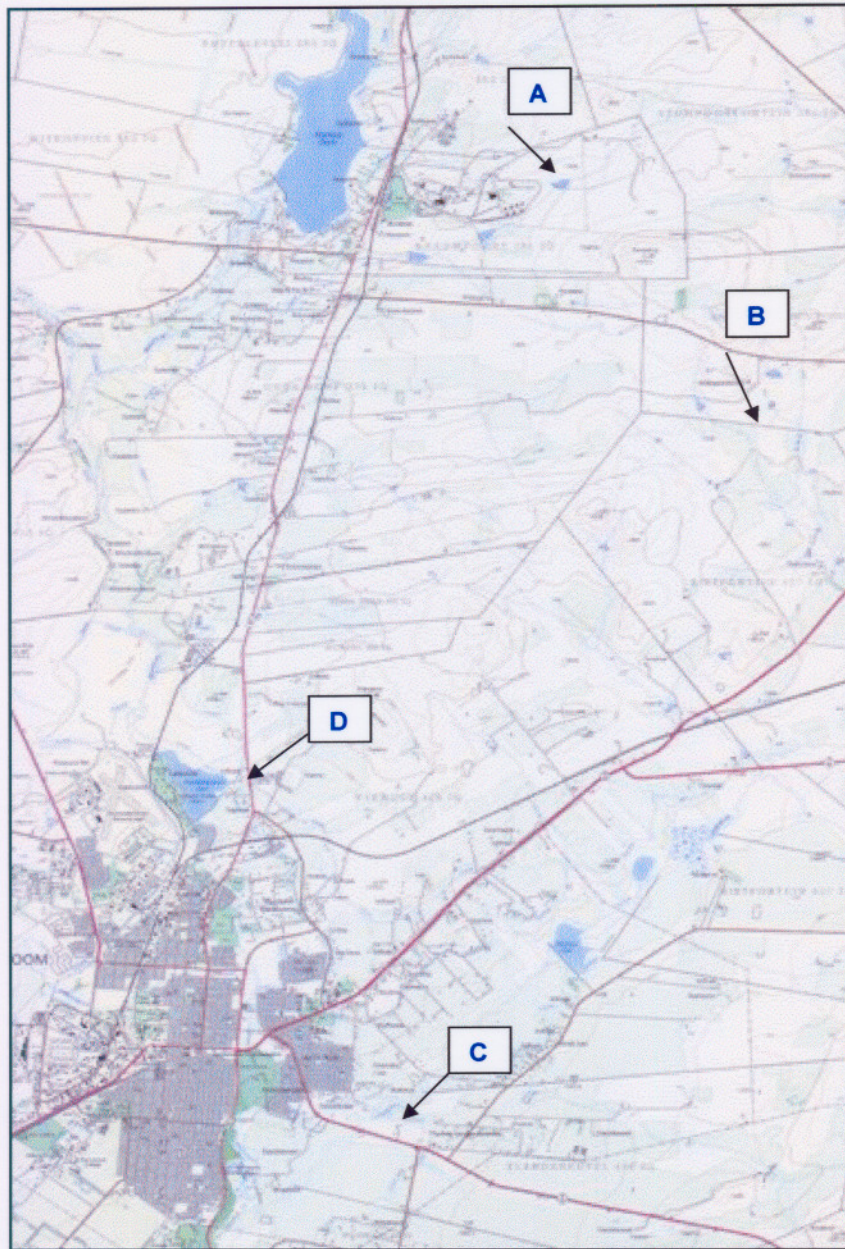
Figure 2.1: Map of the Potchefstroom area showing the sites that were used during the study.

Table 2.1: Physical properties of study site A.

Site A	
Grid reference	26°33'41"S 27°09'35"E
Surface area	14 860 m²
Watershed area	280 ha
Deepest point	104 cm
Source of water	Rainfall
Secci depth	6.5 cm
Vegetation	Aquatic included *Juncus* sp. and *Paspalum* sp. Grassland and wooded thorn trees. No crop fields in catchment.
Other animals at site	Site in a game park. A variety of antelope birds, fish, frogs and crabs are associated with the site.

Table 2.2: Physical properties of study site B.

Site B	
Grid reference	26°35'40"S 27°11'47"E
Surface area	20 500 m²
Watershed area	244 ha
Deepest point	261 cm
Source of water	Rainfall and seasonal fountain
Secci depth	27.5 cm
Vegetation	Aquatic *Paspalum* sp., *Juncus* sp. and *Aponogeton* sp. Surrounded by wooded thorn trees and grassland. No crops in catchment.
Other animals	Cattle, ostriches, fish and crabs and a variety of other frog species share this site.

Table 2.3: Physical properties of study site C.

Site C	
Grid reference	26°44'15"S 27°08'02"E
Surface area	4900 m²
Watershed area	150 ha
Deepest point	190 cm
Source of water	Rainfall as well as water originating from Gerhard Minnebron spring feeds into pond via a canal.
Secci depth	31 cm
Vegetation	Aquatic *Paspalum* sp. *Juncus* sp. and *Aponogeton* sp. Crops (corn) in catchment and grassland directly around pond.
Other animals	Cattle, horses, geese, khoi fish, *Afrana* sp.

Table 2.4: Physical properties of study site D.

Site D	
Grid reference	26°39'53"S 27°06'18"E
Surface area	1350 m²
Watershed area	120 ha
Deepest point	160 cm
Source of water	Rainfall as well as water originating from Gerhard Minnebron spring feeds into pond via a canal.
Secci depth	112 cm
Vegetation	*Cypris* sp. and *Typha* sp. Surrounded by grasslands. No crops in catchment.
Other animals	Cattle, horses, birds and other frog species.

2.2   The Study Animal: *Xenopus laevis*

Table 2.5: Showing the details of the study animal, *Xenopus laevis,* that was used during the study.

Species	The African Clawed Frog, *Xenopus laevis*
Strain	Unspecified
Age	Adult
Number	300 females and 282 males
Source	Three sites in the Potchefstroom region of South Africa

Figure 2.2: Photograph of the male *Xenopus laevis.*

2.3   Collection of Water Samples

From May 2003 until July 2004, water samples were taken every three months at the four study sites. A grab sample was taken at every site in a one-litre solvent-rinsed glass Schott bottle and placed in a cool-box. The bottle was lowered under the surface to a depth of 100 mm and then tilted to allow water to enter. The samples were then transported to the North-West University within 5 hours and stored at 4°C until they

were analysed. The samples were then analysed for atrazine and its metabolites by Mr. Peet Jansen van Rensburg of the Department of Microbiology, School for Environmental Sciences and Development at the North-West University. GLP protocols were followed.

2.4 Climatological Data

At each site, a steel rod was placed in the water as reference marker at which point the water quality parameters were measured. For this purpose, a YSI 556 multi-probe system data logger was used. Measurements were taken for dissolved oxygen (mg/L), conductivity (µS/cm), pH and water temperature (°C). Data recorded were uploaded to a personal computer and processed. Data recorded on the data logger were also filled in on data sheets as a back-up. Climatological data such as rainfall figures, minimum and maximum temperatures, and humidity were obtained from the South African Weather Services weather station situated at Naschem and Potchefstroom and also from owners of the farms on which the site were located.

2.5 Collection and Processing of Samples

On a monthly basis for a period of 14 months, sexually-mature adults were trapped over a 24-hour period using baited bucket traps (Figure 2.3). The method for collecting *X. laevis* is based on the aquatic nature and feeding behaviour of the frog. *X. laevis* rely heavily on their olfactory sense for locating food. Traps were baited with uncooked ox liver. Chunks of liver were placed in gauze bags, to prevent captured frogs from swallowing the bait. Four to six traps per locality were placed in water with 10 to 15 cm protruding above the water surface allowing the frogs to surface for air that entered through holes drilled in the top of the trap. Traps were retrieved between 09:00 and 11:00 the following morning. Male and female frogs were separated and kept in separate containers. Immediately after retrieving the frogs from the traps, ten adult males and ten adult females were randomly selected and immediately anaesthetized in MS-222 (tricaine methanesulfonate). At first, blood samples were collected at the site, but later it was found to be more practical to anaesthetize the frogs in the field and rush them back to the laboratory to complete the procedure. The thorax was opened and a

blood sample collected with an EDTA-rinsed insulin syringe and needle. Blood samples were transferred to EDTA-rinsed Eppendorph vials and kept on ice. Blood samples were centrifuged at 10 000 rpm for 3 minutes. The supernatant was transferred to a labelled cryo vial and the Eppendorph vials with cell component 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 (accuracy 0.1 mm) (Figure 2.4). Frogs were weighed in an empty 600 ml plastic bottle on a Sartorius BP210S balance (0.01g accuracy) (Figure 2.5).

Figure 2.3: Photograph showing a baited *Xenopus* bucket trap set among vegetation with a rock on top to weigh it down.
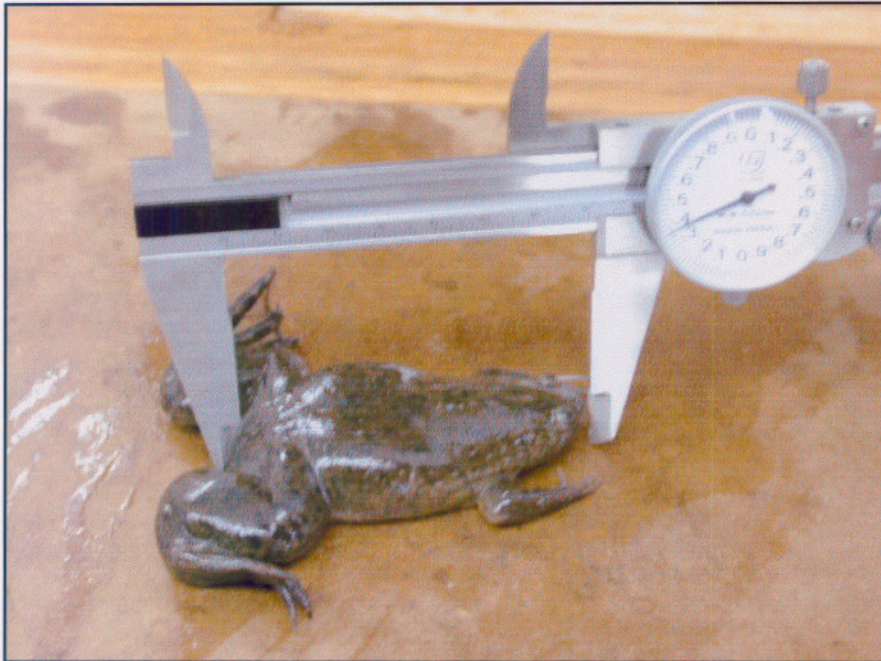
Figure 2.4: Photograph showing the measuring of the snout-vent length using a Vernier Calliper.
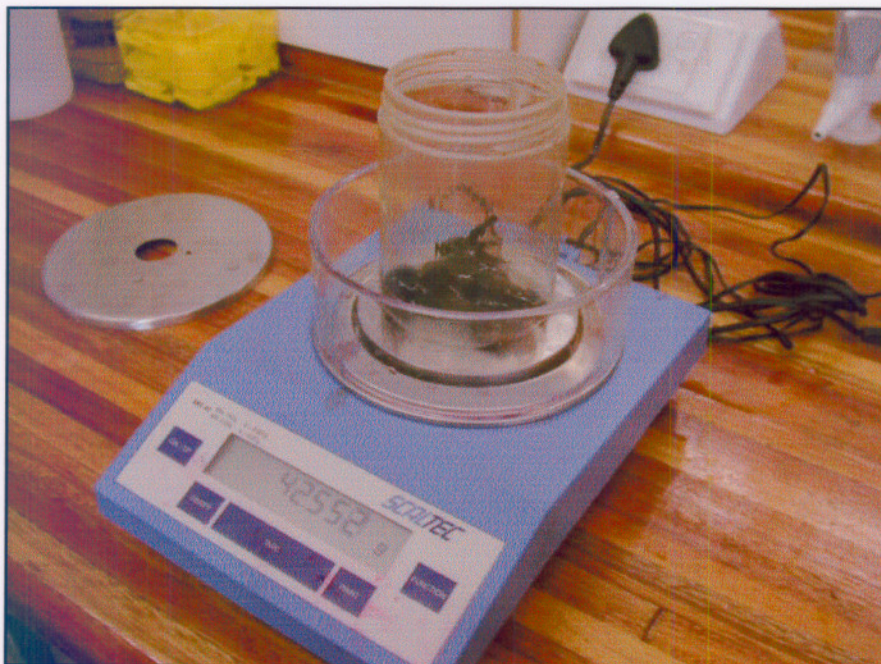
Figure 2.5: Photograph showing the weighing of a frog.

Nuptial pads of males were examined and photographed using a Nikon Coolpix 4500 digital camera attached to a Nikon SMZ1500 dissecting microscope.

The formation of secondary sexual reproductive characteristics is a critical part of the reproductive cycle of *X. laevis*. Classification criteria were developed to characterise the stages of the secondary sexual characters during the life cycle of *X. laevis*. Table 2.6 describes the three classes in which the male nuptial pads were characterised. For females, the cloacal papillae play an important part as a secondary sexual character. The classification for the female cloaca is explained in Table 2.7.

After gross morphological inspection, frogs were dissected and the gonads measured and photographed. Gonads were examined for testicular anomalies and photographed. Gonads were dissected out, measured and weighed. Ovaries were staged according to the criteria in Table 2.8. 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 longest toe of one hind leg was also collected from each specimen and fixed in Bouin's fixative and preserved in 70% ethanol for skeletochronology to determine the age profile of specimens. All carcasses were labelled and frozen.

Table 2.6: Classification of the male nuptial pads.
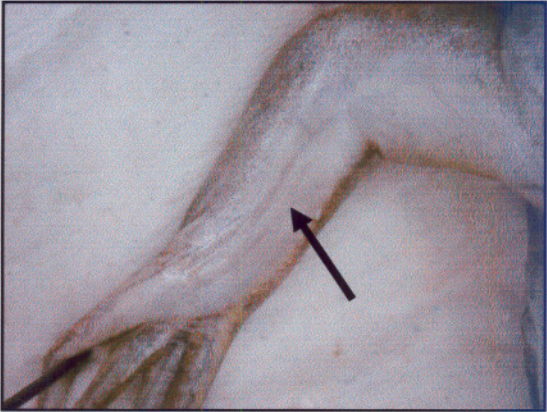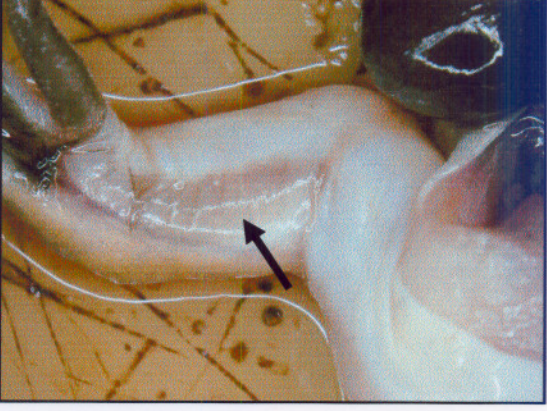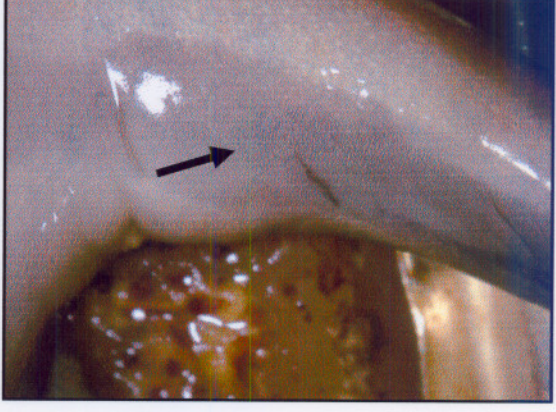
Stage	Description	Photo
1	Hardly visible, pale white in colour.	
2	Distinguishable and shades of grey.	
3	Prominent and dark grey to black.	

Table 2.7: Classification of the female cloacal folds.
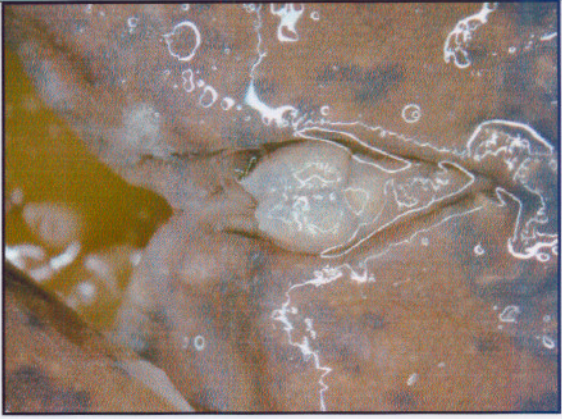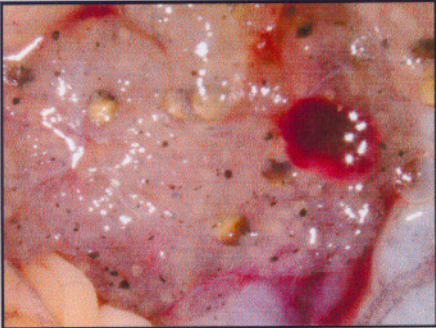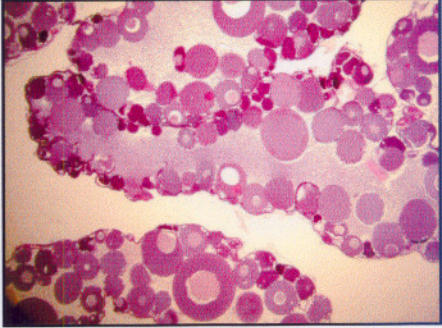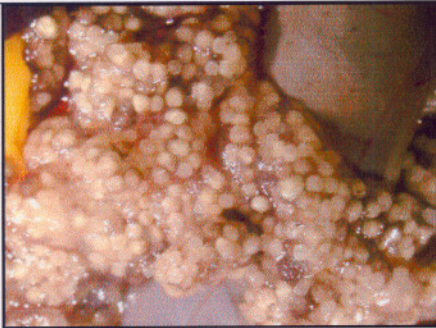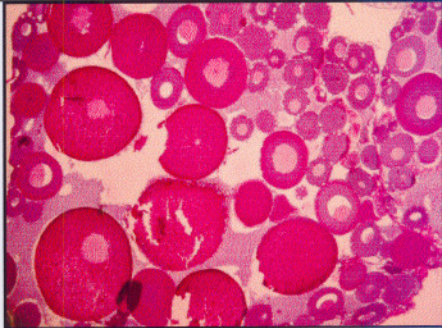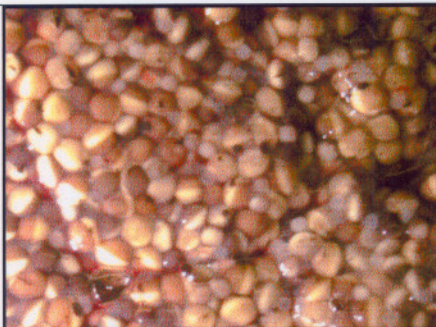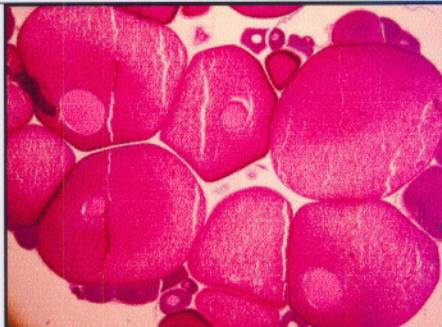
Stage	Description	Photo
1	Small, no swelling or red colouring.	
2	Minimal swelling, but no red colouring.	
3	Maximal swelling and red colouring.	

Table 2.8: Developmental stages of female ovaries.

Stage	Description	Photo	Histological
1	Small granular.		
2	Developing oocytes, majority white in colour.		
3	Oocytes mature with white and dark poles.		

2.6 Histometric Evaluation

Preserved testicular tissue was dehydrated in graded alcohol, embedded in paraffin wax, longitudinally sectioned at 7 µm using a Reichert Jung 2050 microtome (Polzonetti-Magni, 1990; Tavera-Mendoza, 2002 & Jensen, 2001). Sections were stained with Harris haematoxylin and eosin and permanently mounted in DPX mounting medium. Three micrographs per specimen were taken of sections through the anterior, middle and posterior end (Smith *et al*, 2005) of the testis using a Nikon 4500 camera

attached to a Nikon E800 compound microscope (Figure 2.6). Fractional volume and the spermatogenesis stage as well as other tissue types were determined. Each photomicrograph was taken using the 40-X objective lens and saved as JPEG files. The digital images were loaded into a PowerPoint® file. A 7x5-grid overlay was placed on top of each picture. The cells or tissue types under each crossbar was identified and scored (Figure 2.7). All sections were also examined for testicular oocytes and other gonadal deformities.
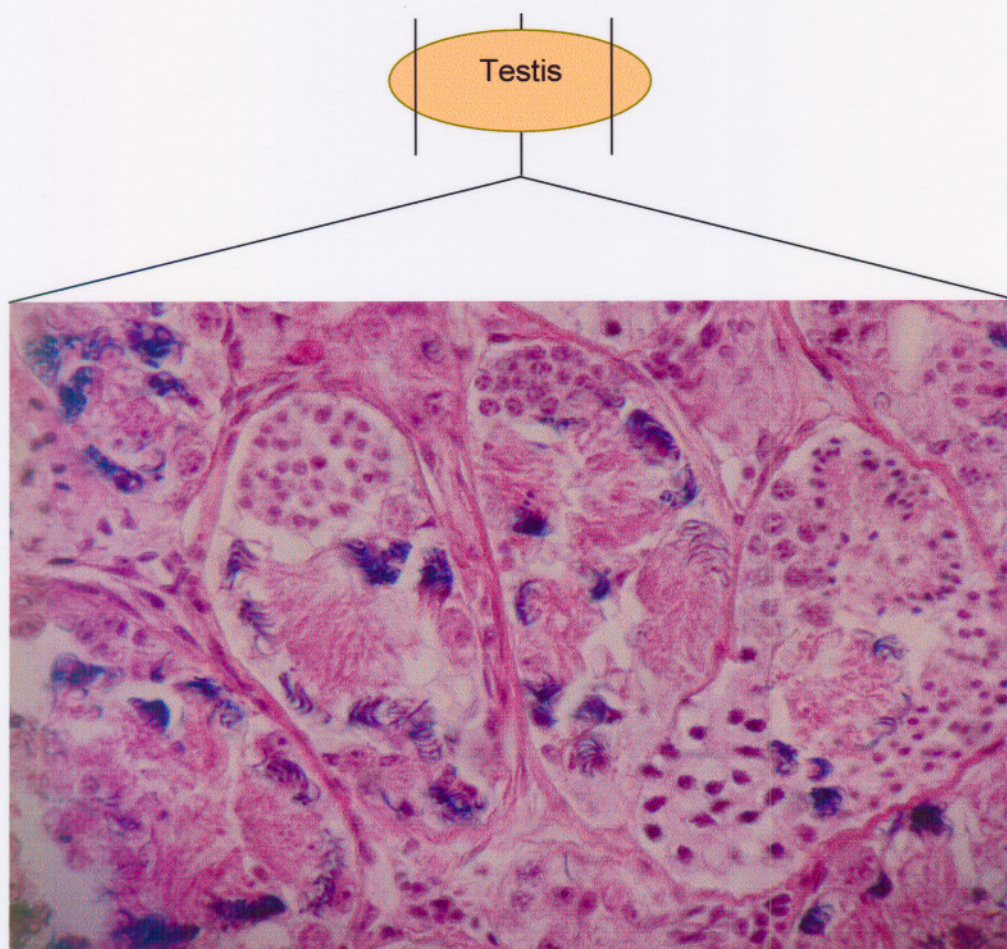
Figure 2.6: Micrograph showing a section through a testis (300 µm in length).

(A)
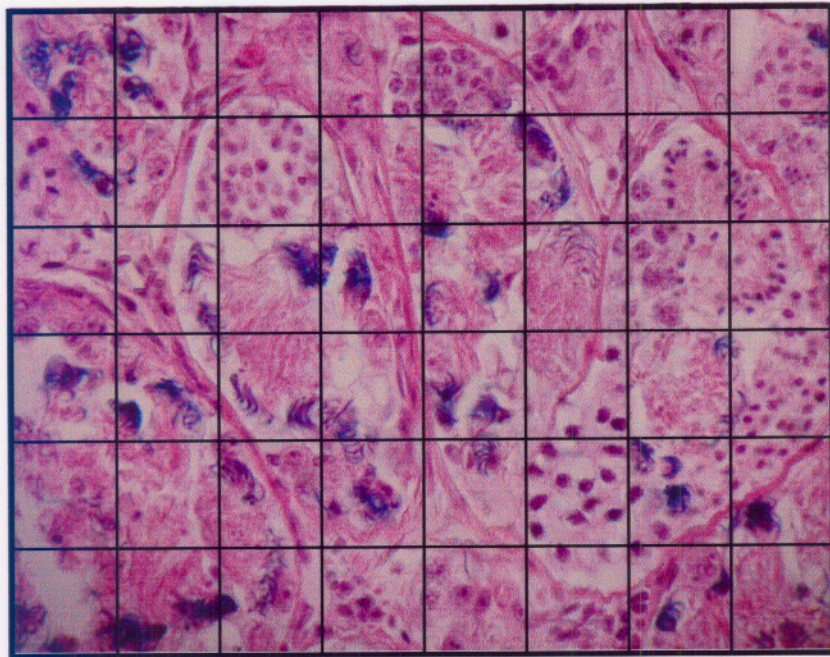
(B)

Figure 2.7: (A) Photomicrograph with the overlay grid and (B) the different types of reproductive cells.

2.7 Skeletochronology

Bone cells that are formed during hibernation in winter are more compact. Tissue formed during the winter is thus denser and growth rings are produced similar to those of a tree. By counting these rings, it is thus possible to determine the age of the frog. The terminal two digits from the longest toe on the one foot of *X. laevis* were removed for skeletochronology. A scalpel was used to cut between the first and second phalanges of the toe. Toes were fixed in Bouin's fixative. After 24 hours, tissue was rinsed in water and transferred to 70% ethanol for storage. Bone was decalcified in Perrenyies solution (Humason, 1987), dehydrated in an alcohol series, cleared in xylene and embedded in paraffin wax using an automated Slee Embedding Center. The toes were histologically sectioned at 7 μm, stained with Gill's haematoxylin and eosin and permanently mounted using DPX mounting medium (Humason, 1987; Cherry, 1992; Bastien & Leclair, 1992; Acker *et al.*, 1986; Hemelaar & Van Gelder, 1980 and Kalb & Zug, 1990).

2.8 Analysis of Plasma for Sex Steroid Hormones

Blood was centrifuged at 10 000 rpm for 5 min at room temperature to separate the plasma fraction. The plasma was collected and stored at -80°C. Frozen blood plasma was shipped to Michigan State University, MI, USA, in a vapour shipper. Plasma samples were extracted twice with diethyl ether. Concentrations of oestrogen and testosterone in blood plasma were measured by competitive ELISA (enzyme-linked immunosorbent assay) as described by Cuisset (1994) and Hecker (2002). In the assay, the plasma steroid competes with acetylchloinesterase labelled steroid for the binding site on polyclonal rabbit anti-serum antibody. Antiserum to T cross-reacted with 5-dihydrotestosterone (46%), 5-dihydrotestosterone (19%), 5-androstane-3,17-diol (3.7%), 11-hydroxytestosterone (3.3%), 5-androstane-3,17-diol (2.7%), 5-androstane-3,17-diol (2.5%), 11-ketotestosterone (0.85%), estradiol (0.54%), 4-androstenedione (0.47%), 4-androstenedione (0.31%), and 17,20P (0.18%) at the 50% displacement level. E2 antibody (Cayman Chemical, Ann Arbor, MI) cross-reacted with estrodiol-3-glucoronide (17%), estrone (4%), estriol (0.57%), T (0.1%) and 5α-dihydrotestosterone

(DHT) (0.1%); all other steroids cross-reacted with the E2 antibody at less than 0.1%. The ELISA was performed using COSTAR high binding plates (Hecker et al., 2005).

2.9 Analysis of Gonads for Aromatase Activity

Analysis of aromatase activity and CYP19 mRNA-concentrations did not form part of the present study, but will be measured following the protocol from Lephart and Simpson (1991) and Sanderson (2000) and will be reported elsewhere.

2.10 Statistical Method

Measured endpoints were evaluated qualitatively and quantitatively and compared in terms of seasonal fluctuations in reproductive status. The programme Sigmaplot® 8.0.2 and Statistica® 7 was used to evaluate the data collected.

2.11 Ecological Aspects of *Xenopus* Reproduction: Case Study

A fourth site, Site D (Table 2.4), was selected to study the population dynamics of *Xenopus*. A mark-and-recapture study was undertaken to determine fluctuations in the population (see Table 2.4). The small pond had a perimeter of 150 m and ten baited *Xenopus* traps were set every 15 m to cover the entire periphery of the pond (Figure 2.8). Every month, the traps were placed in the same trapping positions for a period of three days. Traps were checked on a daily basis and trapped animals were removed, marked and released. The population size was estimated by using the integration of data. This technique has been in use since the 1920s (Woodbury, 1956). The Jolly-Seber Stochastic Method (Donnely et al, 1994) was used for this study.

First, the number of marked individuals at risk on day i (Mi) was estimated using the equation:

$$M_i = m_i + z_i\, r_i\, /\, y_i$$

$$M_i = m_i + \frac{z_i r_i}{y_i}$$

Where: Mi = the number of marked animals caught on day i.

ri = the number of marked animals released on day i.

yi = the number of animals marked before day i that are not caught after day i.

zi = the number of animals marked before day i that are not caught on day i, but are caught after day i.
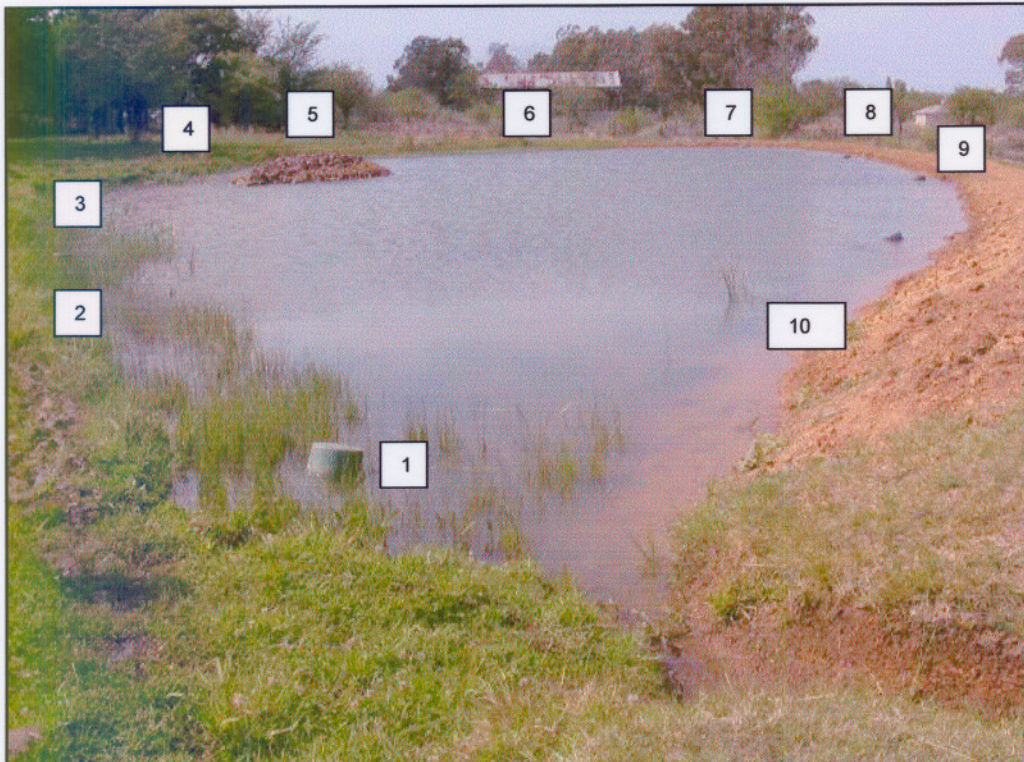
Figure 2.8: Photograph showing the positions of the ten baited *Xenopus* traps that cover the total perimeter of Site D.

Population size (Ni) was estimated as follows:

$$N_i = M_i (n_i + 1) / (m_i + 1)$$

Where: ni = the number of animals caught on day i.

The estimations of survival rate (Øi) and gains (gi) are given by the equations:

$$\phi_i = M_{(i+1)} / (M_i + m_i + r_i) \qquad \text{and} \qquad \hat{g}_i = N_{i+1} - \phi_i N_i$$

The standard error of estimated population size was calculated as follows:

$$SE_{Ni} = \left\{ N_i (N_i - n_i) \left[(M_i - m_i + r_i)/M_i \times (1/y_i - 1/r_i) + 1/m_i - 1/n_i \quad \tfrac{1}{2} \right] \right\}$$

Each frog that was captured, was weighed, measured and branded with an individual number using a branding iron cooled in liquid nitrogen (Figure 2.9).

Figure 2.9: Photograph showing the branded number on the ventral surface of a recaptured *Xenopus laevis*.

Tadpoles were collected using a 50 cm x 50 cm steel frame that was placed in the water and all tadpoles inside were collected and staged according to Nieuwkoop and Faber (1956). During nightly visits, the water was screened for frogs in amplexus. Evidence of spawn was noted. Other amphibian species as well as predators were identified and noted.

2.12 Obstacles Encountered

Identifying suitable sites with an absence of atrazine was difficult. Since atrazine contamination is possible through atmospheric deposition it was quite difficult to locate atrazine-free sites. Drought during spring and the beginning of summer in November, caused study site A to dry up from December 2003 to February 2004 with the result that no frogs could be collected during this period. This also happened at site D where it was dry during January and September 2004 when the owner let the water out to clean the pond.

2.13 Quality Control and Quality Assurance

This study was conducted in the spirit of Good Laboratory Practice Standards and Quality Assurance programme guidelines. Quality Assurance inspections were performed by Dr Keith Solomon from Guelph, Canada and Mr Tom Gale from Syngenta to insure the integrity of the study. A copy of all data, the protocol and the final report is being kept at the testing facility.

CHAPTER 3

RESULTS:

SEASONAL FLUCTUATIONS IN THE REPRODUCTIVE CYCLE
OF *XENOPUS LAEVIS*

3.1 ENVIRONMENTAL DATA

3.1.1 Water levels

The summer of 2004 was very dry. Site A dried up completely from December 2003 to February 2004 (Figure 3.1) while the water level of site B dropped drastically (Figure 3.2). This, however, did not disrupt the sampling at site B. Sites C and D received water via an irrigation canal and sampling was possible throughout the study except for the months of January and September 2004 when the owner drained the water from site D to clean out the pond.

Steel rods were used as markers to determine the depth of the water and where water quality was measured. The steel rods were completely outside the water body during the dry period and then completely covered by water during the wet period and thus, could not be used effectively during the study. Measurements were taken in line with these markers.

Figure 3.1: Photograph of Site A that dried up from December 2003 to February 2004.

Figure 3.2: Photograph of Site B that indicates a reduced water level during the spring of 2003.

3.1.2 Water Analysis

The detection limit for atrazine was 0.01 µg/L. The results of this study (SA01-E) and that of the previous study (SA01-A) are given in Table 3.1.

Table 3.1: Atrazine concentrations from the study.

Site	SA01-A (Previous study)	SA01-E (2004)
A	≤ 0.01 µg/L	0.11 µg/L
B	≤ 0.01 µg/L	≤ 0.01 µg/L
C	Not analysed	2.13 µg/L
D	Not analysed	3.91 µg/L

3.1.3 Climatological Data

The year 2003 had a very dry period until November 2003 when more that 100 mm of rain was recorded. December was again a very dry month, but exceptionally good rain was recorded during February and March 2004 (Figure 3.3). The pattern of a dry December fits in with the 10-year mean, but this year was one of extremes. The average daily temperature was comparable with the 10-year mean and reached a peak during December 2003 and January 2004 (Figure 3.4). The same phenomenon was noticed for water temperature at the three sites. Water temperature did not vary much between the three sites and the water reached a maximum temperature between November and December 2003. The temperature at site C remained higher during January and February 2004, while the temperature at site B dropped (Figure 3.5). The daily relative humidity is a relatively-good indication of wet and dry periods during the study as it reflects the atmospheric conditions. The maximum and minimum daily relative humidity was low during September and December 2003 and corresponds with the rainfall (Figure 3.6). The pH at the three sites varied little and fluctuated between 6.5 and 8.8 through the study period (Figure 3.7). The conductivity at site C differed significantly from that of site A and B. Conductivity at site A and B was fairly stable (≤ 100 µS/cm), while site C had high values of 200 – 700 µS/cm (Figure 3.8). Dissolved

oxygen decreased gradually from September 2003 and reached a minimum during November 2003 at all three sites and then started rising again until May 2004 (Figure 3.9).
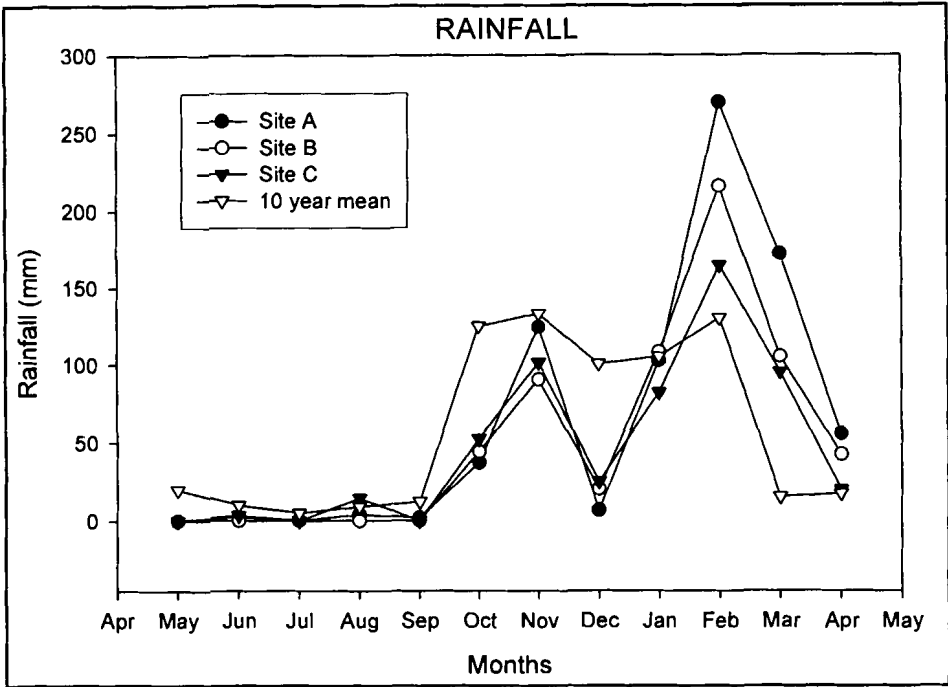
Figure 3.3: Rainfall recorded between May 2003 and April 2004 at the three different study sites.
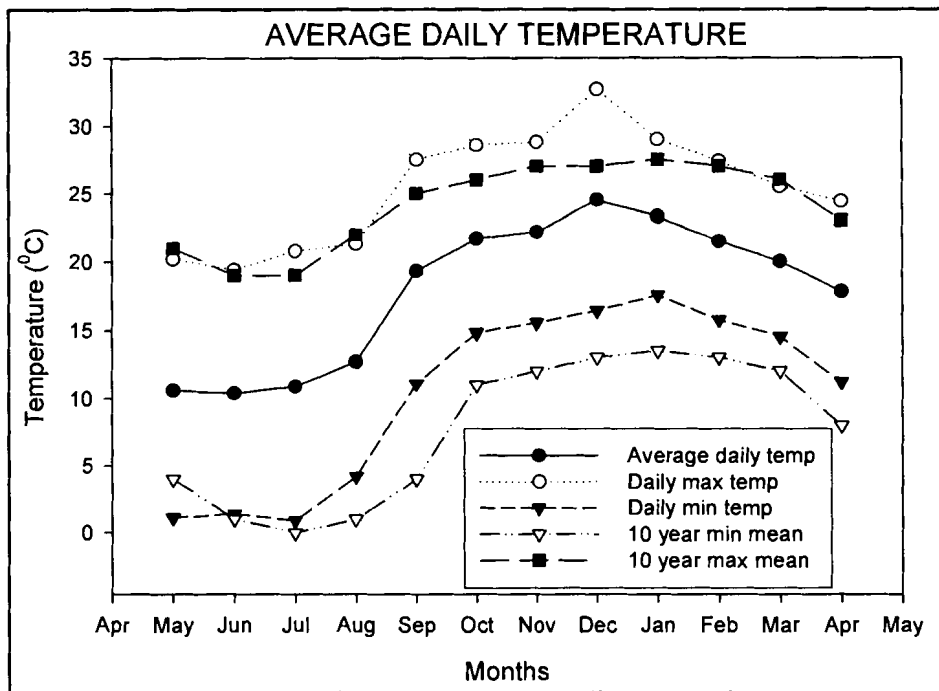
Figure 3.4: Average daily temperature, daily maximum temperature and daily minimum temperature for the study period and the 10-year mean.
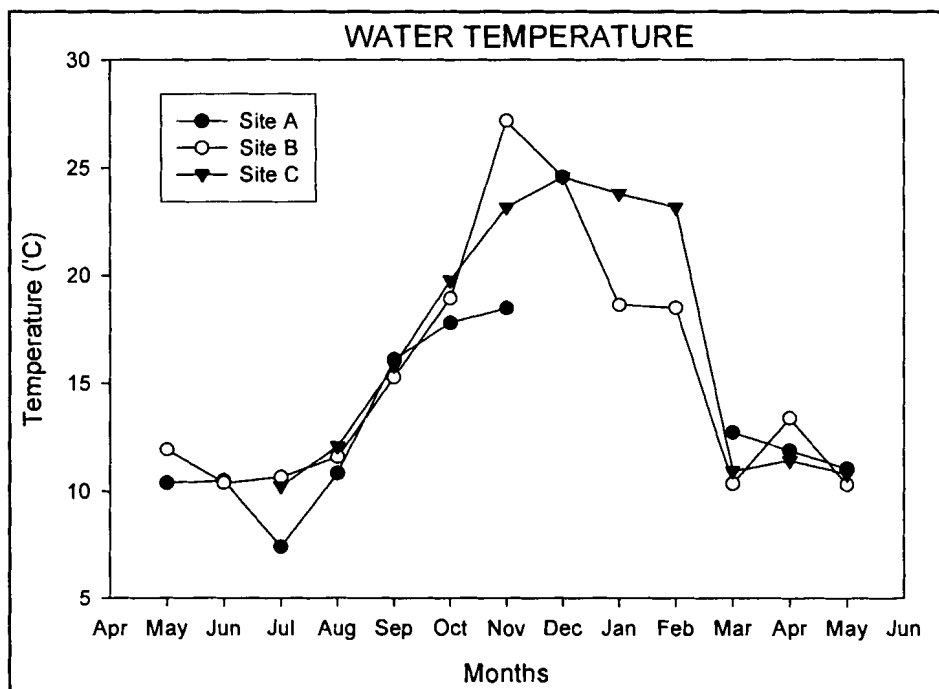
Figure 3.5: Recorded water temperature at the three different sites between May 2003 and May 2004. The gap in the data for site A represents the period in which the pond dried up.
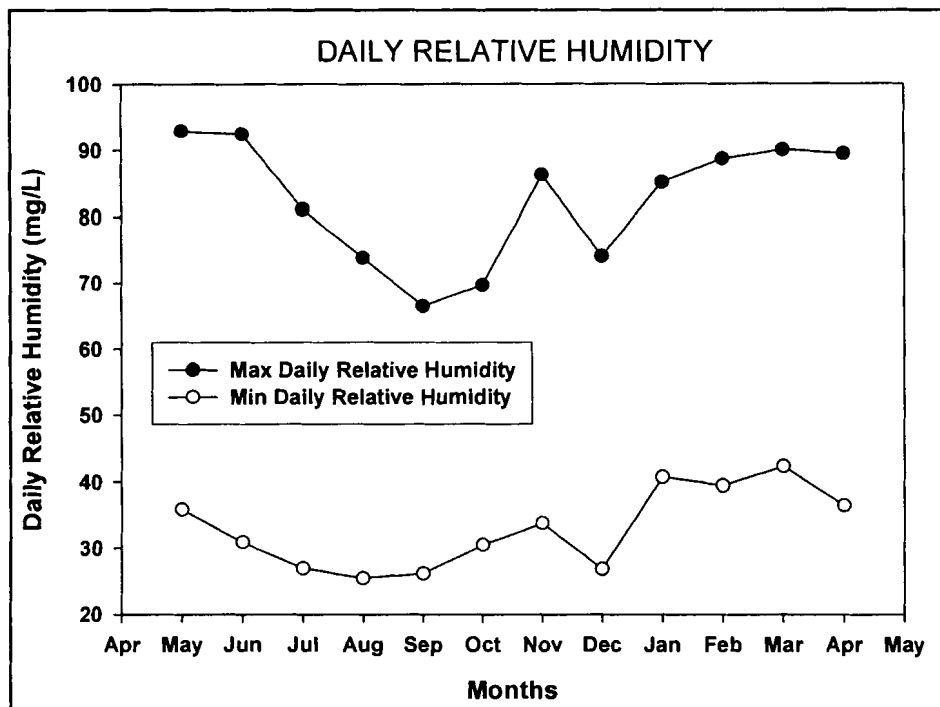
Figure 3.6: Daily minimum and maximum relative humidity between May 2003 and April 2004.
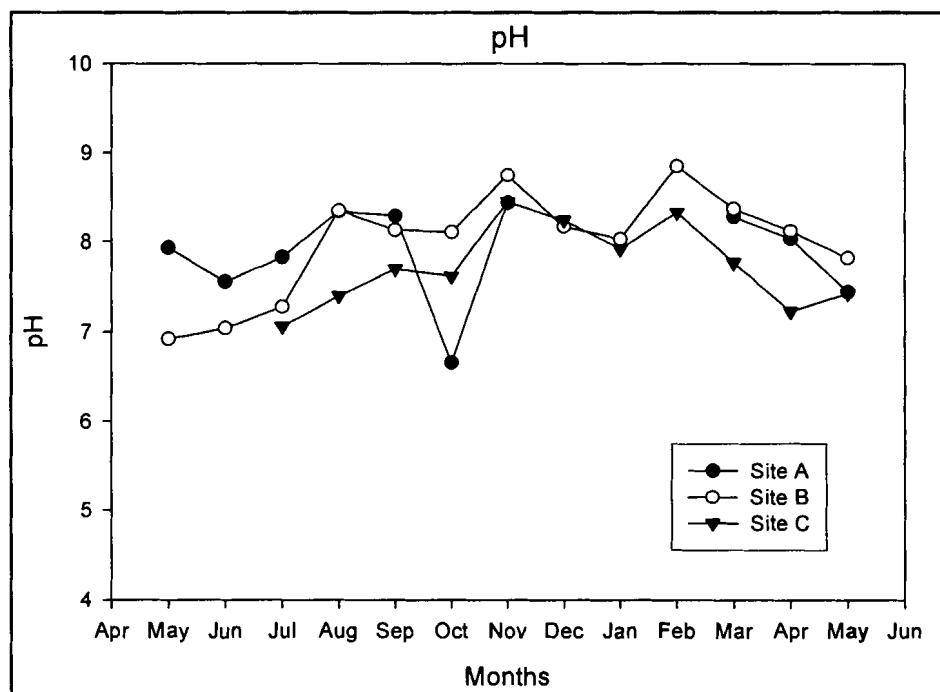
Figure 3.7: The pH at the different sites between May 2003 and May 2004.
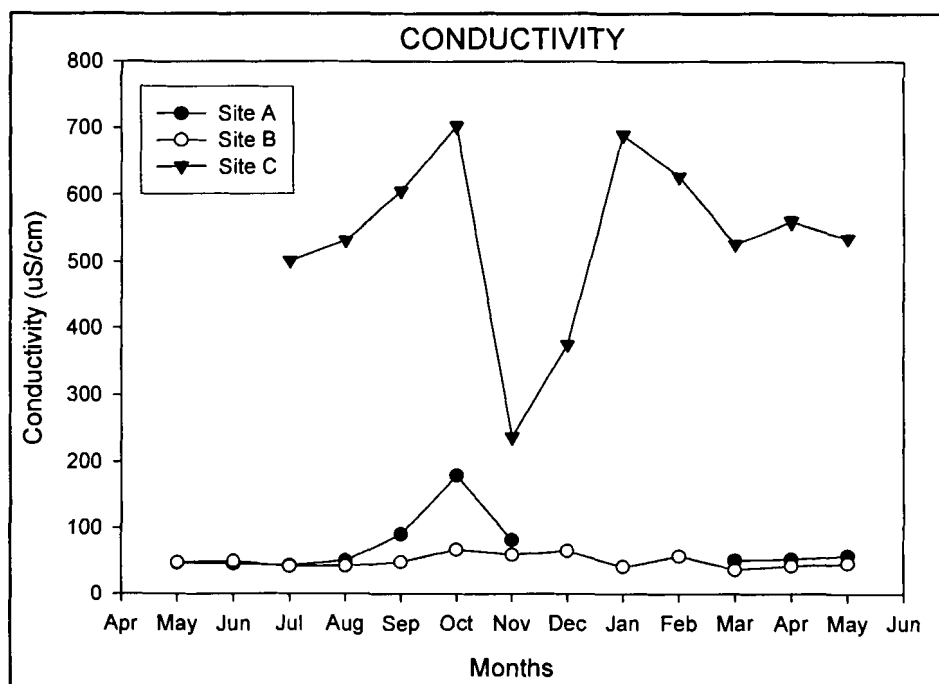
Figure 3.8: Conductivity at the three study sites between May 2003 and May 2004.
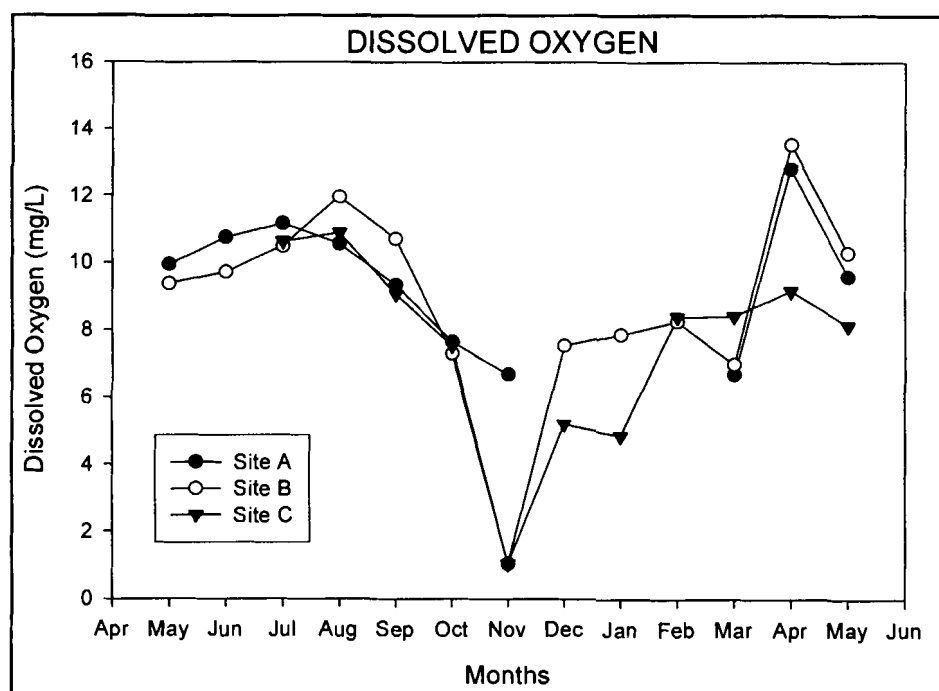
Figure 3.9: Dissolved oxygen at the study sites between May 2003 and May 2004.

3.2 EXTERNAL SEX CHARACTERS AND GONADAL DEVELOPMENT

3.2.1 Frogs Collected

The target was to collect ten adult male and ten adult female *Xenopus laevis* per month for the period May 2003 to May 2004. It became more difficult to collect the required number of frogs closer to the end of the study. The total number of frogs that was collected over the study period is given in Table 3.2.

Table 3.2: Number of *Xenopus laevis* captured at each site during the study.

SITE	A		B		C	
SEX	Male	Female	Male	Female	Male	Female
TOTAL	84	85	104	110	94	105

3.2.2 Snout-Vent Length

The minimum, maximum and mean snout-vent length (SVL) of the male and the female *X. laevis* collected at the three sites are given in Table 3.3. This shows that the females were significantly larger than the males. This is also reflected in Figure 3.11, which clearly indicates a difference in size from the three study sites combined. The SVL of the females ranged between 41 mm and 114 mm, while the SVL of the males ranged between 46 mm and 85 mm.

The respective SVL for males and females at the three sites are given in Figure 3.10 (A) and (B). This gives a clear indication of the fluctuations among different *X. laevis* populations. All three sites showed a decline in SVL for males and females towards the end of September 2003, after which an increase was observed to January 2004 for the male SVL (Figure 3.11A). The female SVL (Figure 3.10B) showed a decline towards January 2004. The male SVL declined to beyond January 2004 at site B and site C, but the male and female SVL at site A showed an increase during March 2004, whereafter it decreased for the males.

Male SVL showed a normal distribution (p ≤ 0.00040), while the same was true for the female SVL (p ≤ 0.00001) for the three study sites. The Kruskal-Wallis ANOVA-test (comparing of multiple independent samples) was used to evaluate the variance between the three study sites. The test showed that there was no significant differences between the mean SVL of the male or the female frogs from the three study sites (males p = 0.376, and females p = 0.064).

Table 3.3: Minimum, maximum and mean snout-vent lengths of the frogs at the sites.

	MALES			FEMALES		
Site	Mean	Min	Max	Mean	Min	Max
A	66.7	50.5	81.3	73.0	50.4	114.4
B	61.4	46.0	84.4	74.3	41.8	113.3
C	62.0	47.3	85.2	73.5	48.2	110.2

(A)

AVERAGE MALE SNOUT-VENT LENGTH
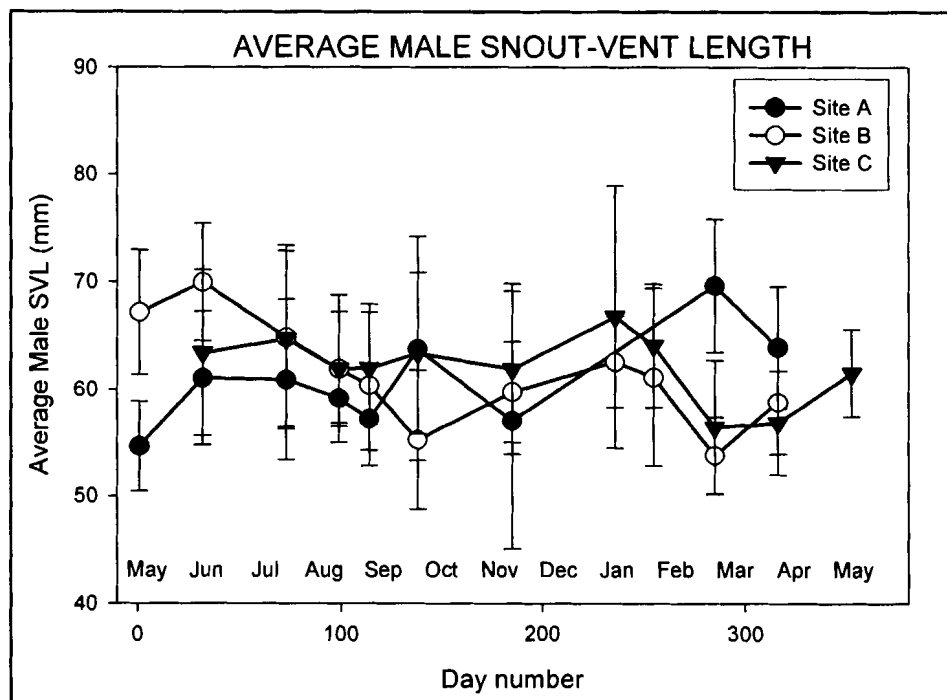
(B)
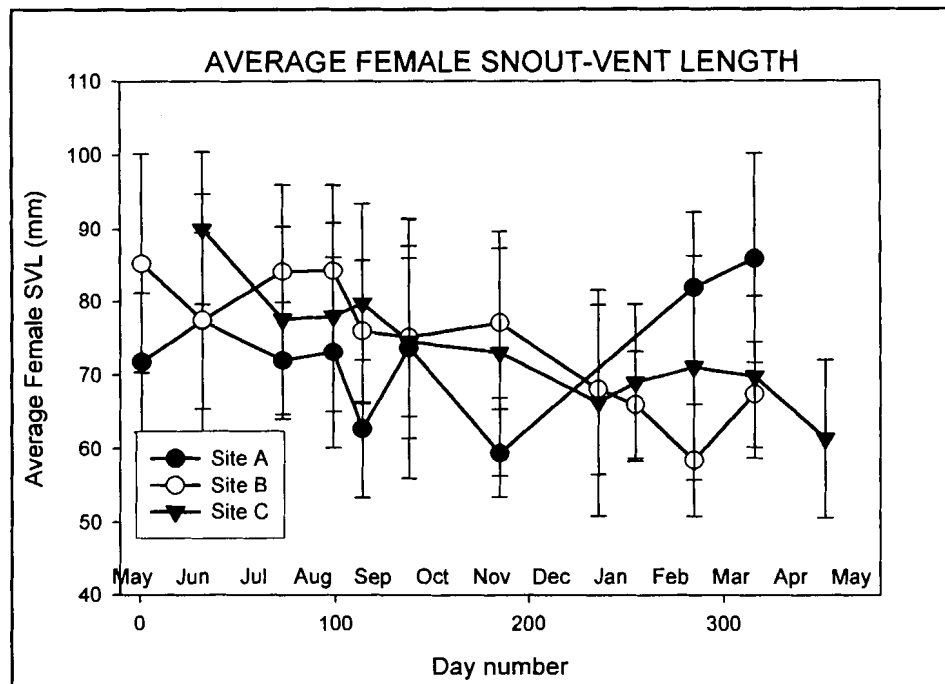
Figure 3.10: The average snout-vent length and standard deviation of (A) male and (B) female *Xenopus laevis* captured during the study period from May 2003.
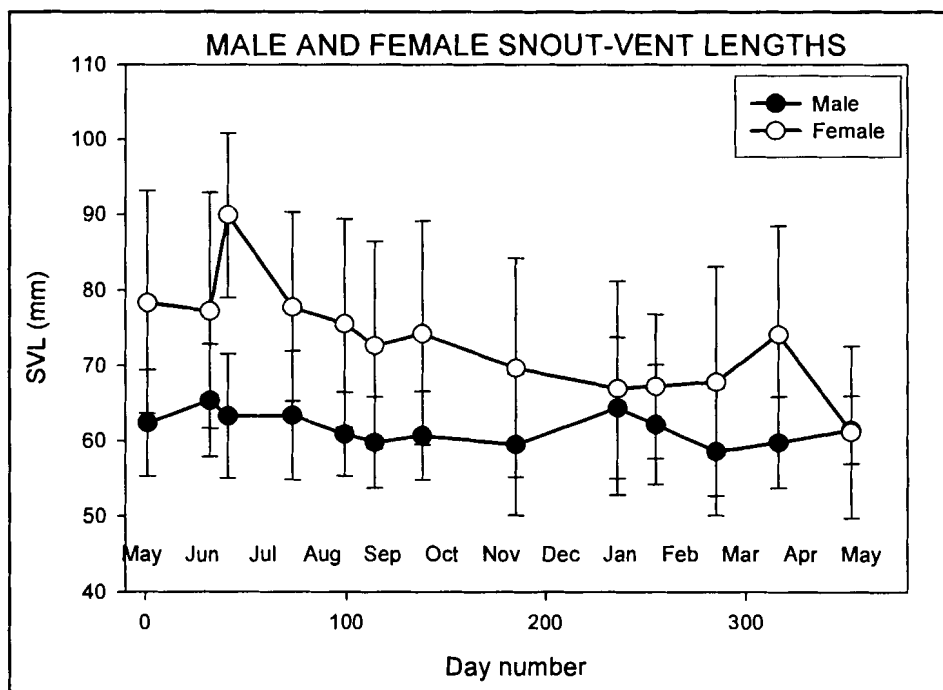
Figure 3.11: Graph showing the average snout-vent length for males and females at the three study sites with the standard deviation.

3.2.3 Mass of the Frogs

The minimum, maximum and mean mass of the male and female *X. laevis* caught at the three sites are given in Table 3.4 and the mean values with standard deviation are shown in Figure 3.12 (A) and (B). Combining all the sites, the females had a minimum mass of 12.9 g and a maximum of 155.1 g, while the males had a minimum mass of 7.5 g and a maximum of 74.7 g. These data show that females are significantly heavier than males in size (Figure 3.13).

Mean mass of the frogs varied over time and between the three study sites. Frogs at site A showed a peak in body mass during March 2004, after which it decreased. The mean mass of frogs at sites B and C showed a gradual decrease from November 2003 and January 2004, respectively.

The mean mass of the frogs collected from the three sites, were normally distributed between the three study sites (male $p \leq 0.00001$ and female $p \leq 0.00001$). The Kruskal-Wallis ANOVA-test was used to evaluate the variance between the three study sites. The test showed that there was no significant differences between the mean mass of the male or the female frogs from the three study sites (males $p = 0.826$, and females $p = 0.124$).

Table 3.4: Minimum, maximum and mean mass of the frogs at each study site.

	MALES			FEMALES		
Site	Mean	Min	Max	Mean	Min	Max
A	35.6	16.5	74.7	63.8	12.9	118.4
B	27.8	8.0	74.5	49.9	13.1	155.1
C	28.6	7.5	63.5	50.3	13.1	131.6

(A)
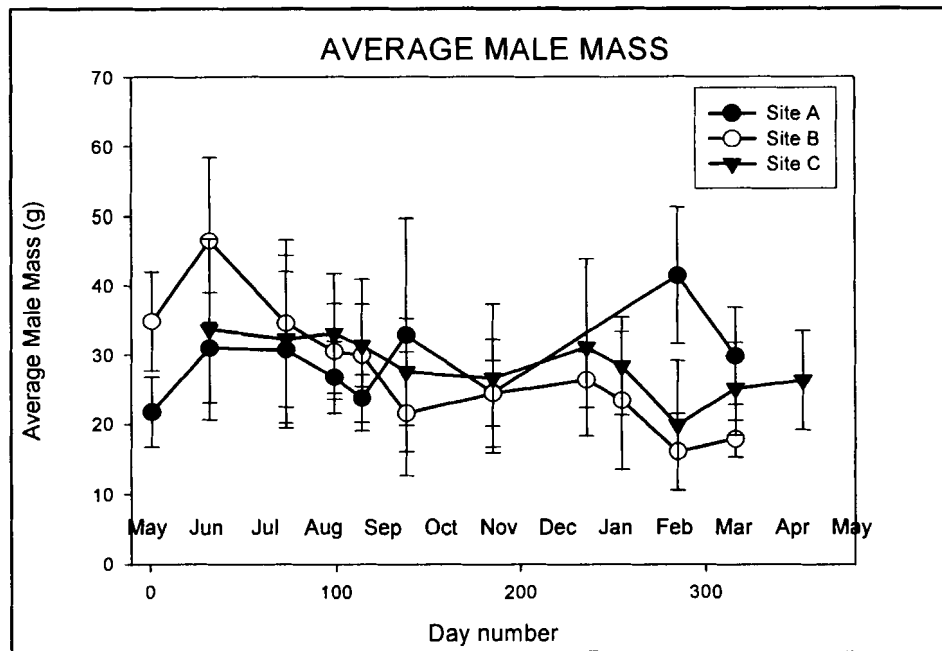
(B)
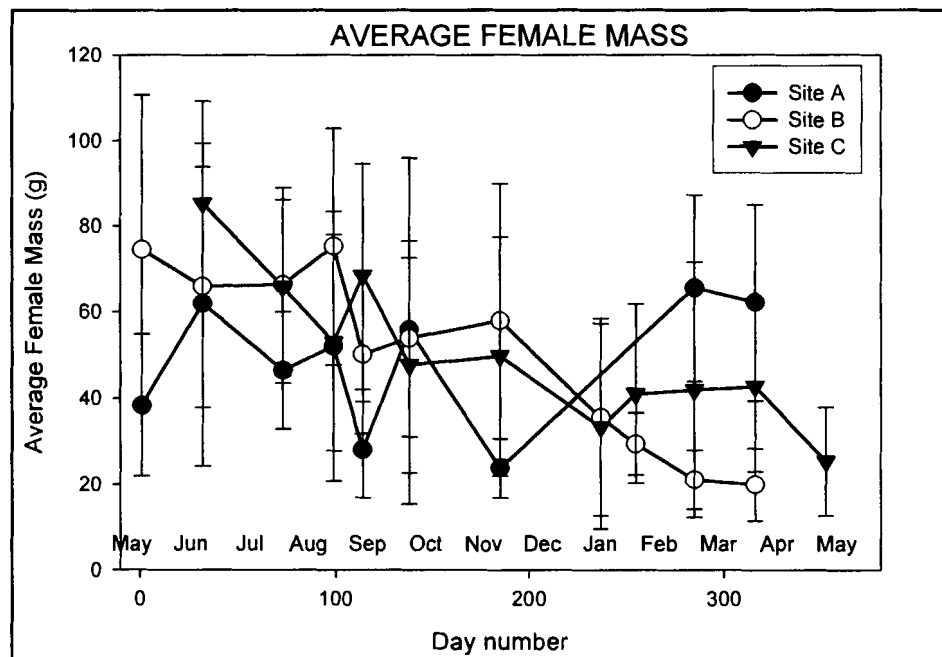
Figure 3.12: The average mass and standard deviation of (A) male and (B) female *Xenopus laevis* during the study period from May 2003.
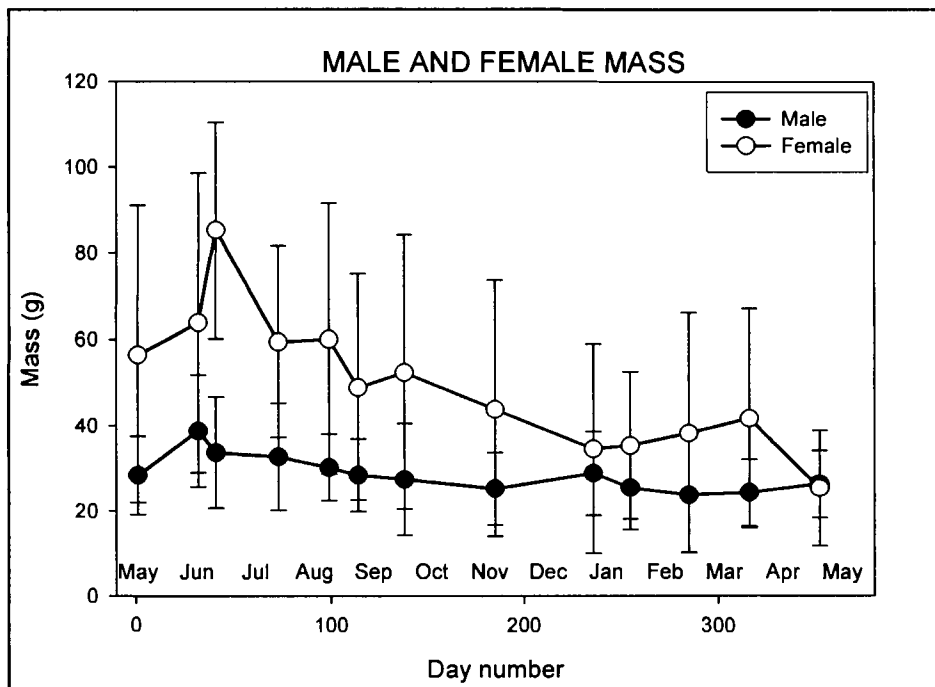
Figure 3.13: Graph showing the mean masses of the male and female frogs at the three study sites combined with the standard deviation.

3.2.4 Gross Morphology of the Gonads

The gross morphology of the gonads in the frogs varied between the three sites. Site A (Figure 3.14) showed an increase towards March / April 2004, while sites B and C showed a sharp decrease during the same months. All three study sites showed an increase in mean testes lengths from the end of September 2003 and a decrease towards November 2003. Mean testis width (Figure 3.15) showed an increase from the beginning of the study towards October 2003, after which it decreased during November 2003 and then increased again towards January / February 2004. Thereafter it decreased again towards April / May 2004. Figures 3.16 gave an indication of the fluctuations between study sites. Mean testes mass increased towards October 2003, whereafter it decreased during November 2003. Site B is the only site that may indicate an increase towards November 2003. The mean testes mass then increased for sites A and B towards February 2004, whereafter it decreased again. The average testes mass at site C dropped towards February 2004, but increased during March 2004.

The mean mass of the female ovarian tissues (Figure 3.17) showed less variation between sites. The mean ovarian weight for site A increased during October 2003, while it remained low at sites B and C. Then the mean ovarian weight at site A declined towards November 2003. Sites B and C suddenly showed an increase and then decrease towards January 2004 and thereafter remained low. Site A again showed a high ovarian mass during April 2004, while sites B and C remained constantly low.

Normal distribution analysis was performed on the length, width, and mass of the left and right testes, as well as the mass of the left and right ovaries. No significant differences were found between the three study sites ($p \leq 0.048$ and $p \leq 0.00001$). The Kruskal-Wallis ANOVA-test was used to evaluate the variance between the three study sites. The test showed that there was no significant differences between the mean testes length ($p = 0.239$), mean testes width ($p = 0.751$), or the mean testes mass ($p = 0.861$) of the male at the three study sites. The same was true for the mean ovarian mass of the female frogs ($p = 0.076$).
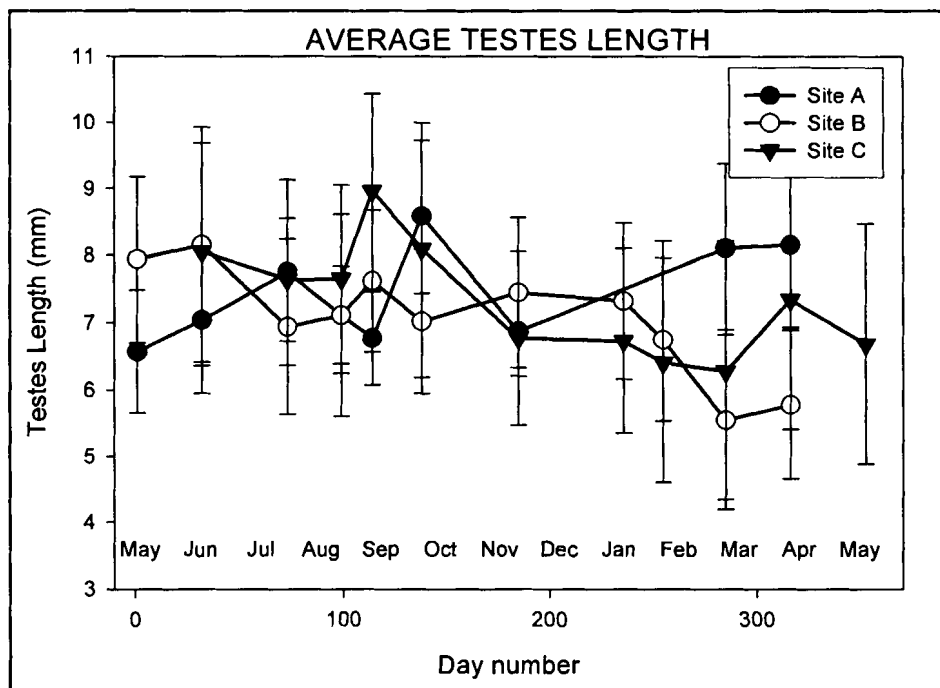
Figure 3.14: Mean lengths of the testes in frogs from three experimental sites.
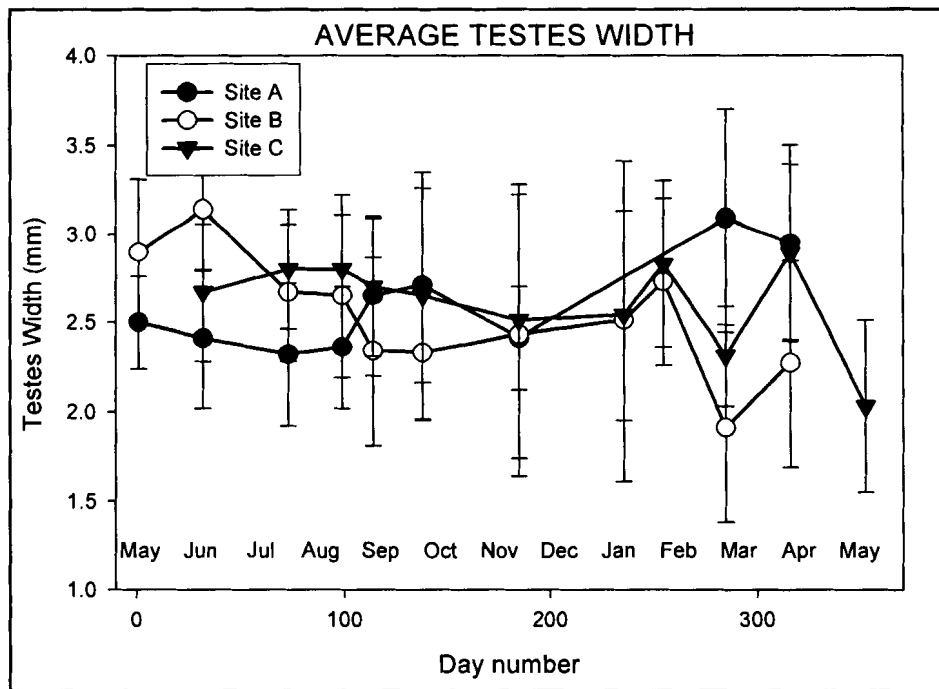
Figure 3.15: Mean widths of the testes in frogs from three experimental sites.
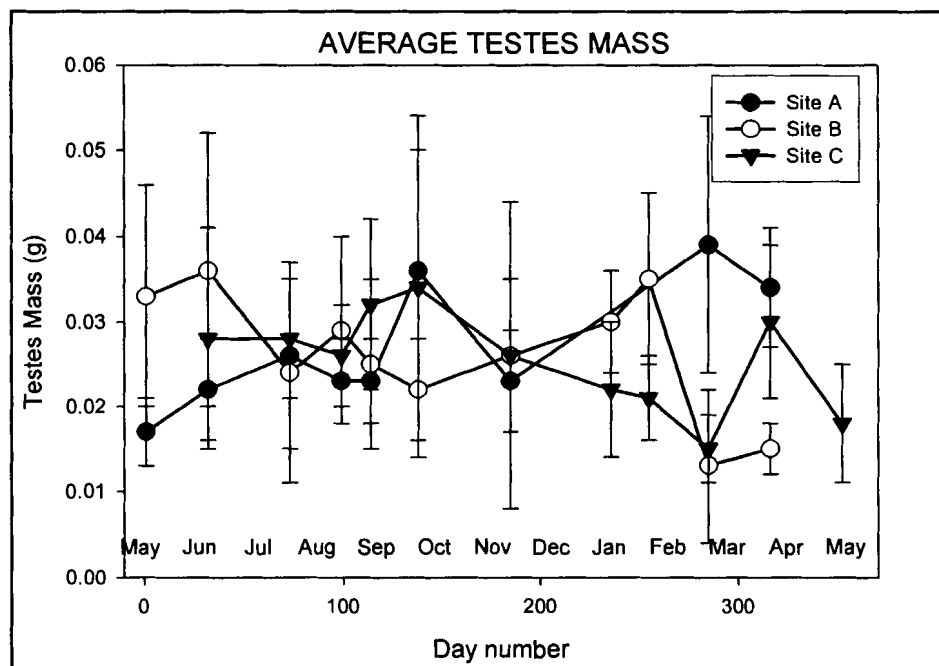
Figure 3.16: Mean mass of the testes in frogs from three experimental sites.
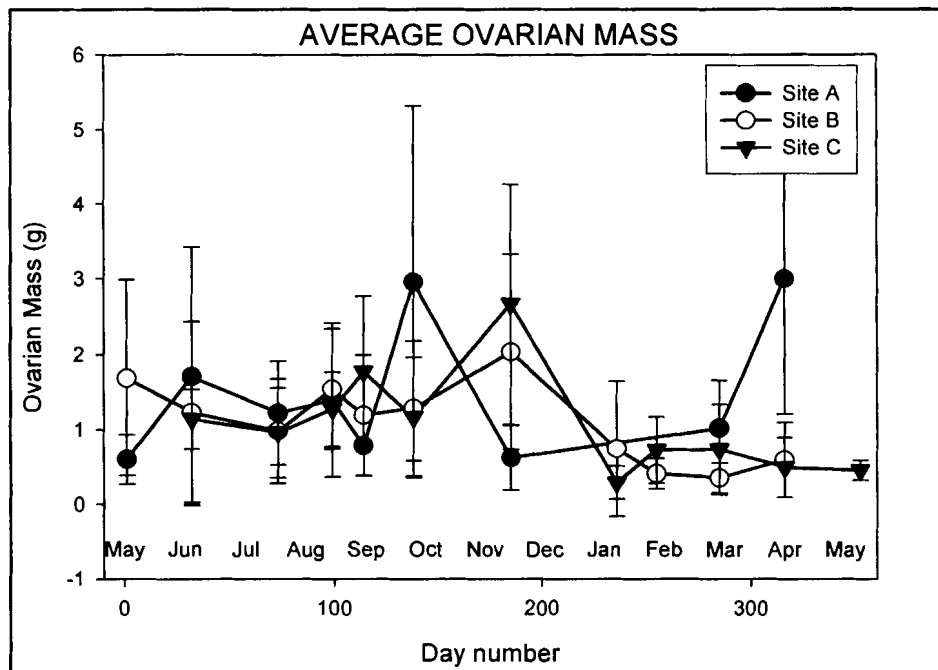
Figure 3.17: Mean mass of the ovaries in frogs from three experimental sites.

3.2.5 Gonado-Somatic Index

The mean gonado-somatic indexes (GSI), which is an expression of the gonad mass as a percentage of the body mass, for all three study sites were combined in one graph (Figure 3.19). It is clear, but also obvious, that the female GSI was much greater than the male GSI. It is also clear from the graph that there was an increase towards October / November 2003, whereafter a decrease in GSI is visible towards January 2004. This is emphasised by Figure 3.18. There appeared to be an increase from August 2003 towards October 2003. The male GSI (Figure 3.18 A) then declined during November 2003. The female GSI (Figure 3.18 B) for sites B and C only showed an increase towards November 2003, whereafter the GSI decreased towards January 2004. Male GSI was normally distributed (p ≤ 0.00001) between the three sites. The same was true for the female GSI (p ≤ 0.00001). The Kruskal-Wallis ANOVA-test was used to evaluate the variance between the three study sites. The test showed that there was no significant differences between the mean GSI of the male frogs or the female frogs from the three study sites (males p = 0.260, and females p = 0.056).

(A)
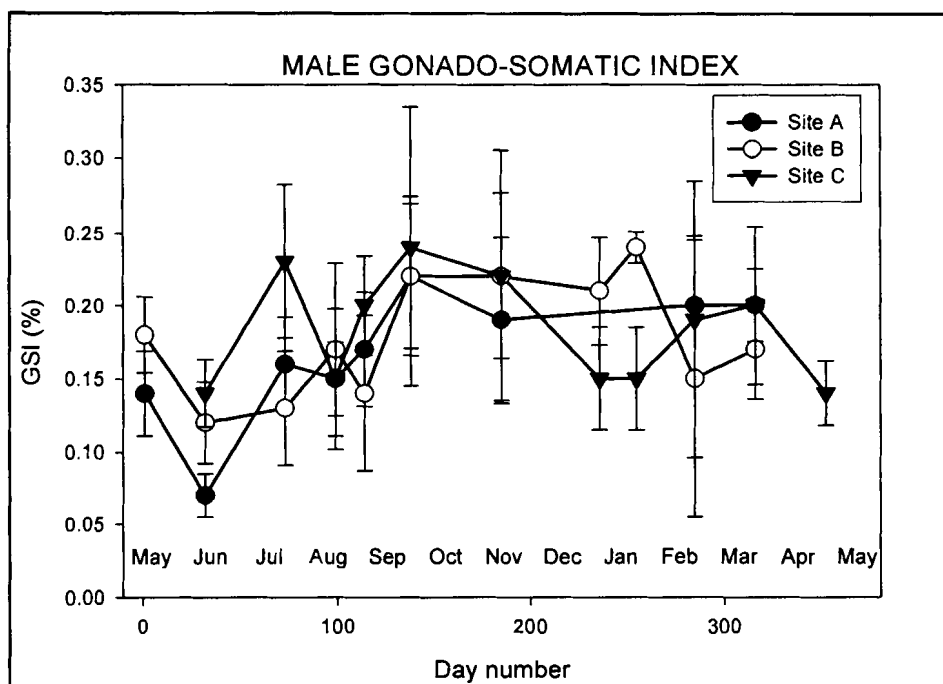
(B)
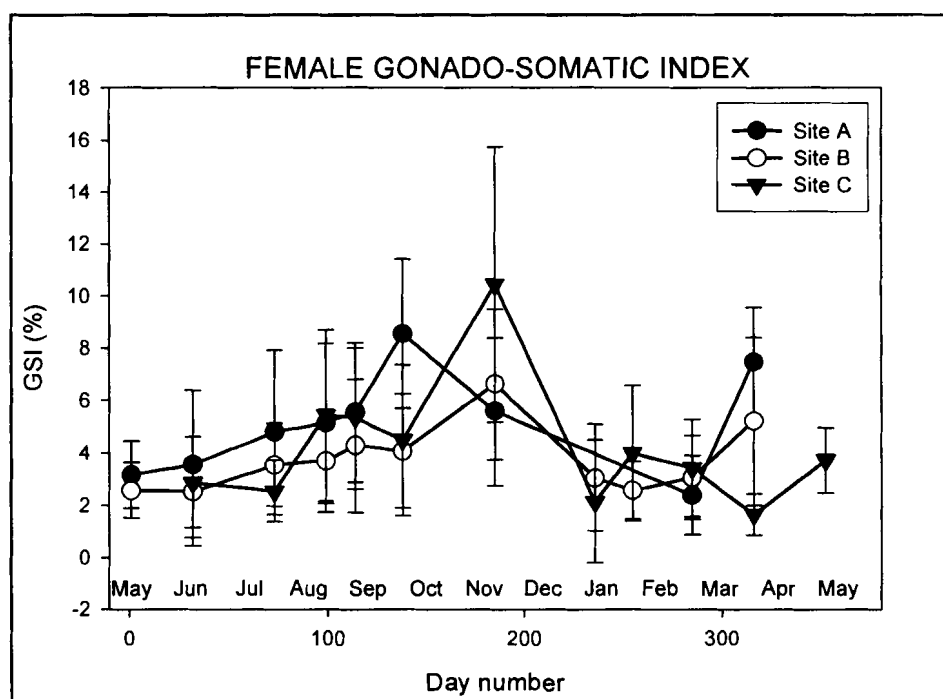
Figure 3.18: Mean GSI for (A) males and (B) females from May 2003.

50
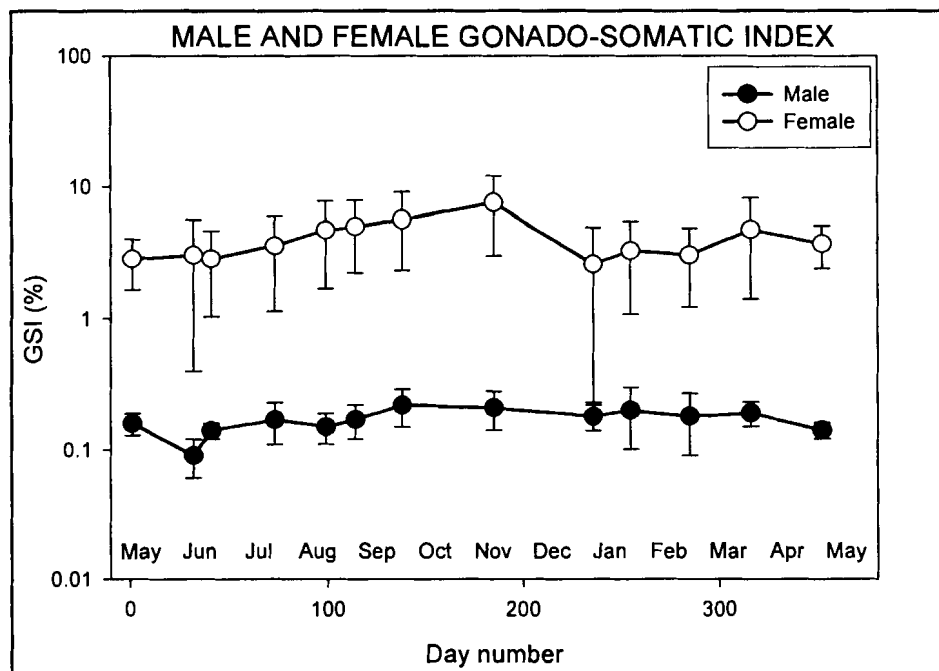
Figure 3.19: Graph showing the combined gonado-somatic index of the three study sites for males and females.

3.2.6 Staging of Ovaries

Figure 3.20 shows the combined development of the female ovaries at the three study sites. From this graph, it is clear that well-developed ovaries (stage 3) are found throughout the year. Number of frogs with stage 3 ovaries did, however, peaked in November 2003. It is also interesting to note that there was also stage 1 and stage 2 ovaries present throughout the year, even in the winter months. Figure 3.21 shows the variation in ovarian development at the three study sites. Site A showed maximum development of the ovaries during November 2003, while site B peaked during October 2003 and site C during September and November 2003.

GENERAL OVARIAN DEVELOPMENT
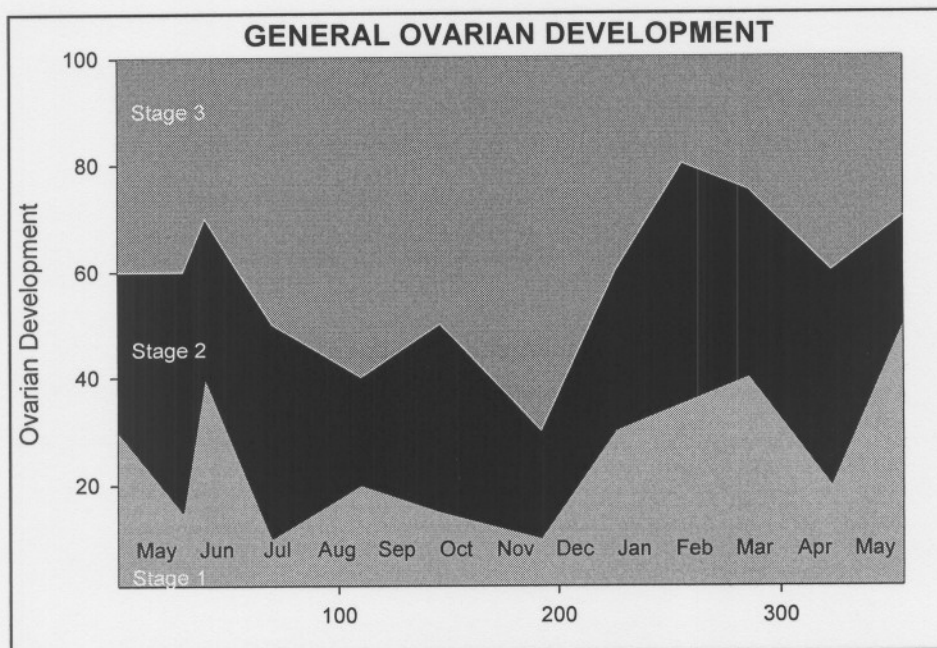
Figure 3.20: Graph showing the female ovarian development for all three study sites.

(A)

OVARIAN DEVELOPMENT AT SITE A

(B) OVARIAN DEVELOPMENT AT SITE B
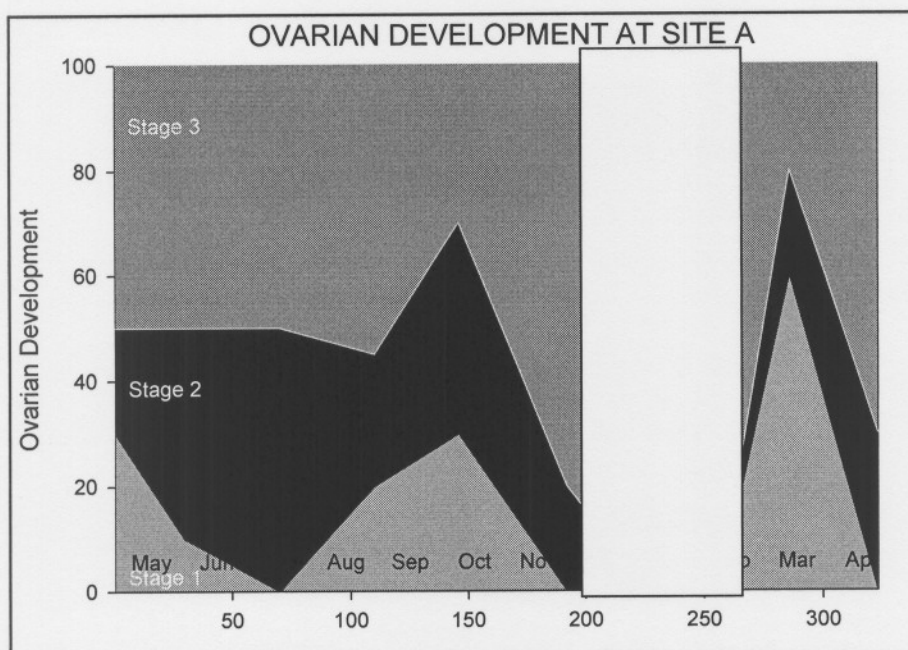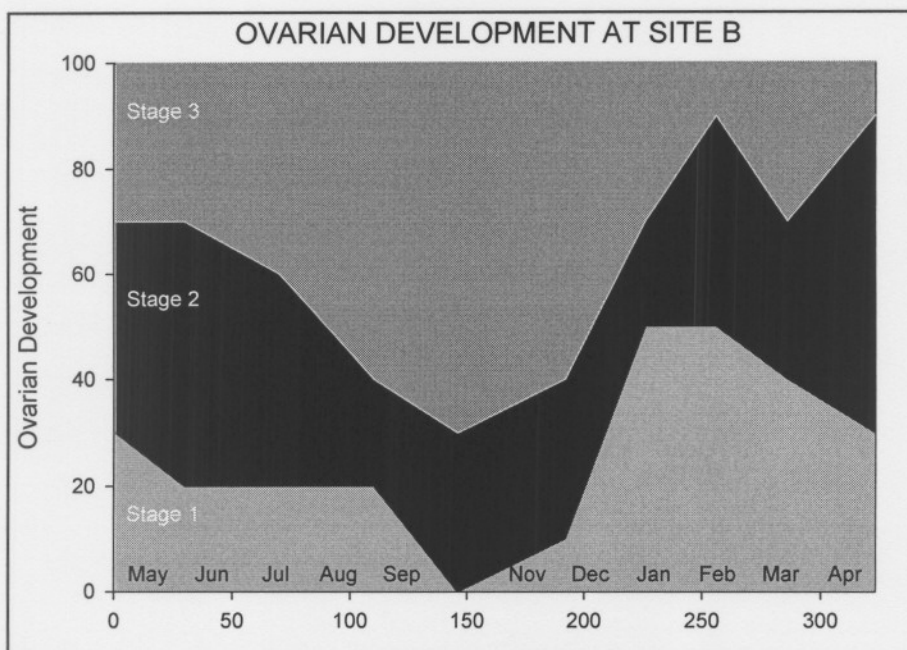
(C) OVARIAN DEVELOPMENT AT SITE C
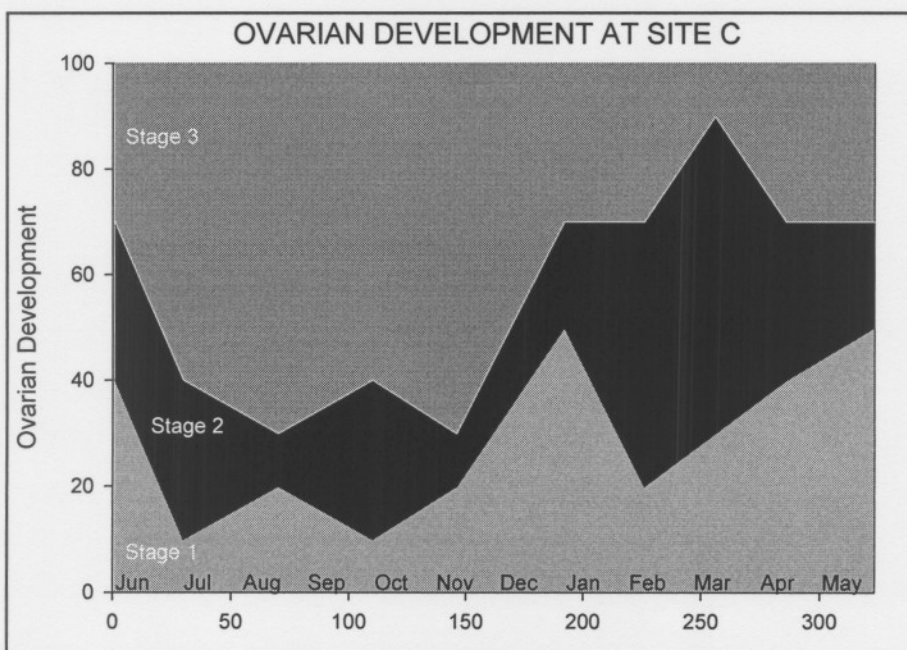
Figure 3.21: Graphs showing the variation in ovarian development at (A) Site A (dry period indicated with white block), (B) Site B and (C) Site C during the study period.

3.2.7 External Sex Characters

Nuptial pads

A clear seasonal pattern was observed for the presence of nuptial pads (Figures 3. 22 and 3.23). The prevalence of stage 3 nuptial pads reached a peak during the month of January 2004. The nuptial pad development was slow to start but could be clearly seen as a more focused process during a specific time of the year. Figure 3.23 shows the variation in nuptial pad development between the three study sites. Sites A and B showed more frogs with nuptial pad development during November 2003, while site C only peaked during January 2004.
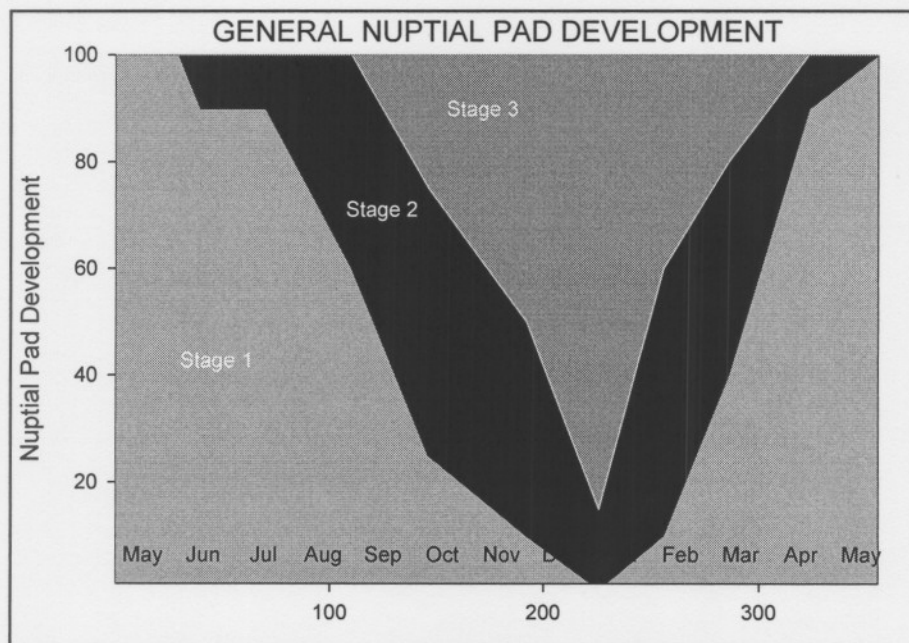
Figure 3.22: Graph showing the nuptial pad development for males for the three study sites.

(A)

NUPTIAL PAD DEVELOPMENT AT SITE A

Stage 3

Stage 2

Stage 1

(B)

NUPTIAL PAD DEVELOPMENT AT SITE B

Stage 3

Stage 2

Stage 1

(C)
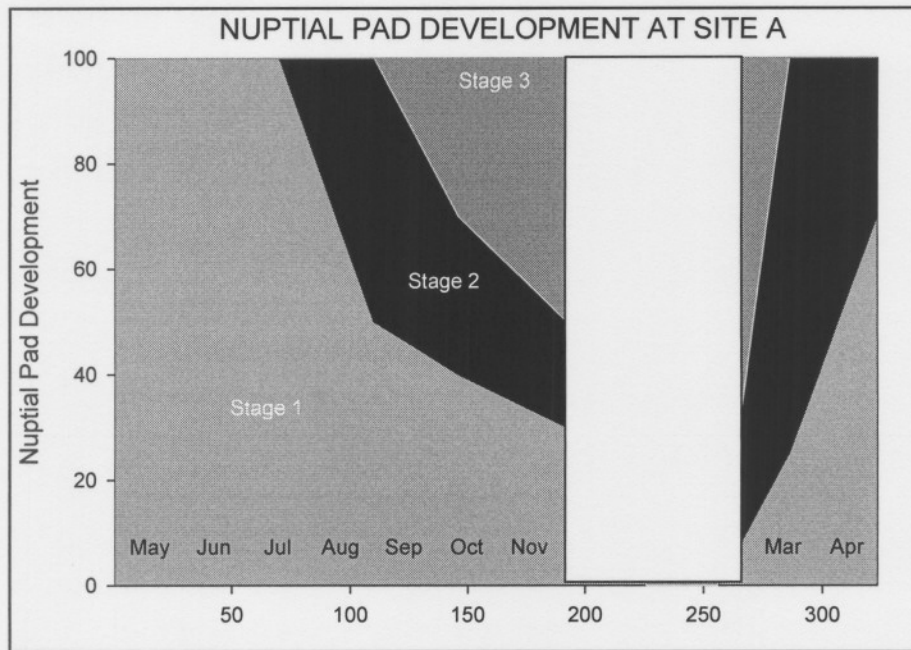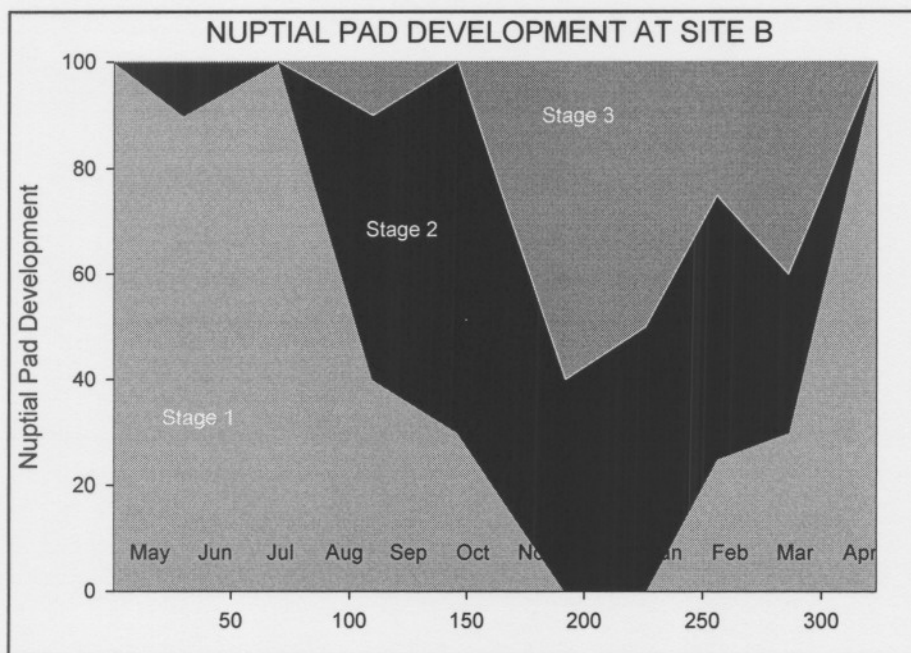
NUPTIAL PAD DEVELOPMENT AT SITE C
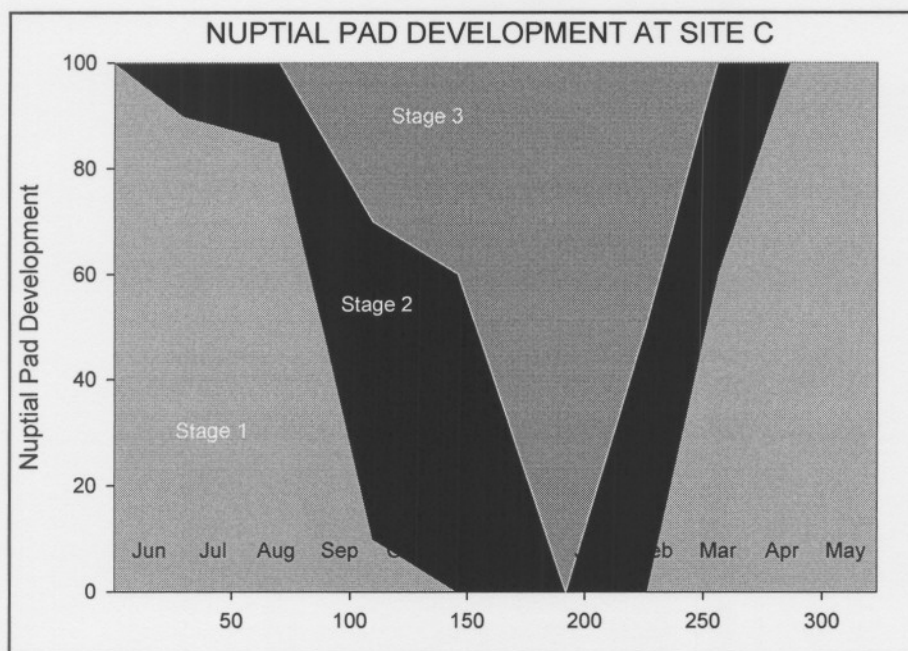
Figure 3.23: Graphs showing the variation in nuptial pad development at (A) Site A (dry period indicated with white block), (B) Site B and (C) Site C during the study period.

Cloacal folds

When considering all three study sites (Figure 3.24), is it clear that maximum cloacal development occurred during November 2003. There were swollen cloacal folds present throughout the year, but red, swollen folds (stage 3) were only observed from September 2003 to April 2004. The variations between the three study sites are seen in Figure 3.25. Site A showed maximal cloacal development during October and November 2003 and then again during March 2004. The cloacal development at site B reached a maximum during November 2003, while site C showed a peak during October 2003.
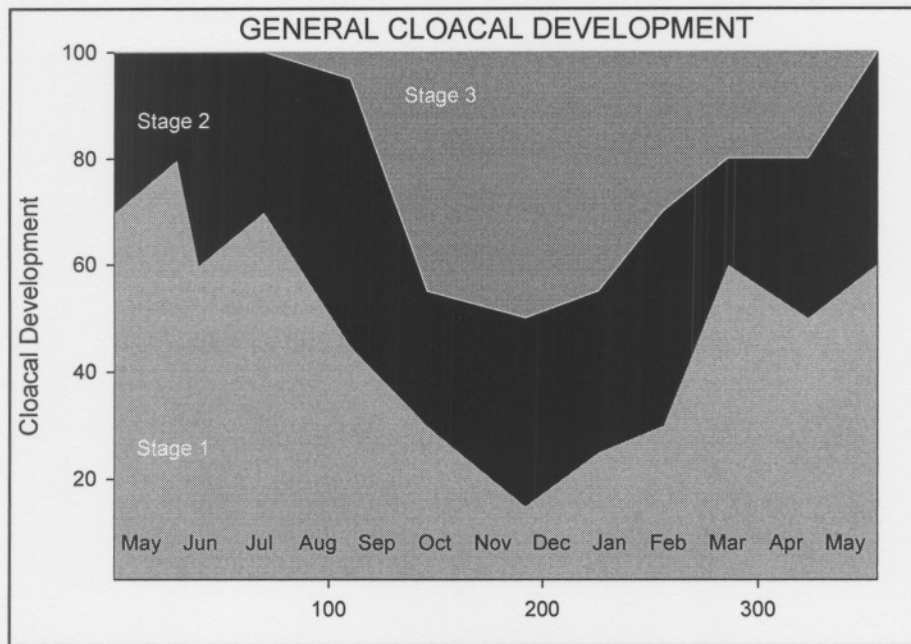
Figure 3.24: Graph showing the female cloacal development at the three study sites.

(A)
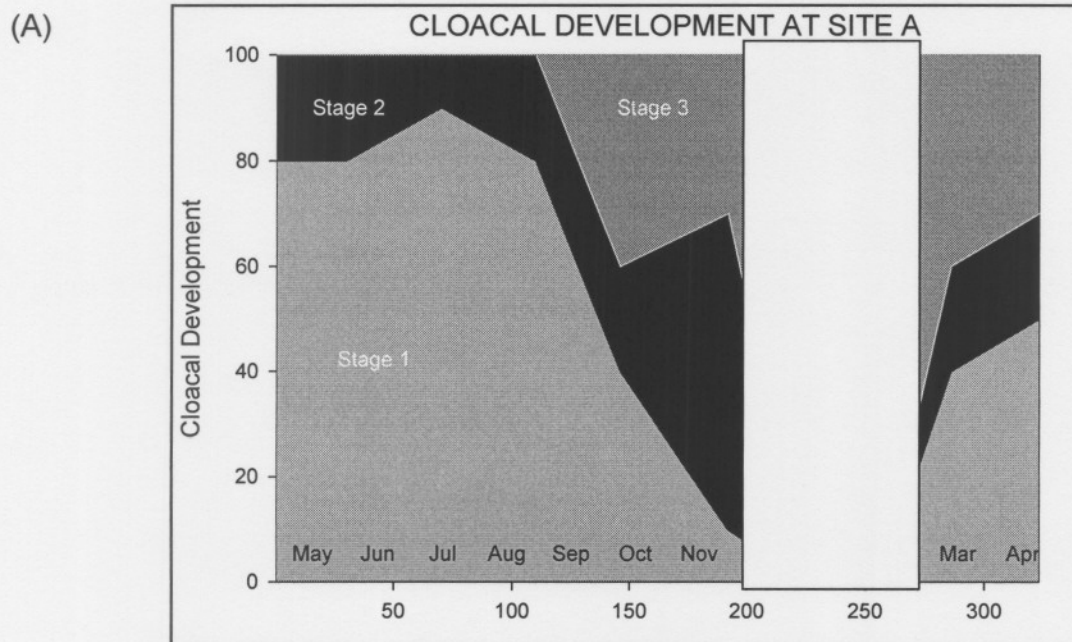
(B)
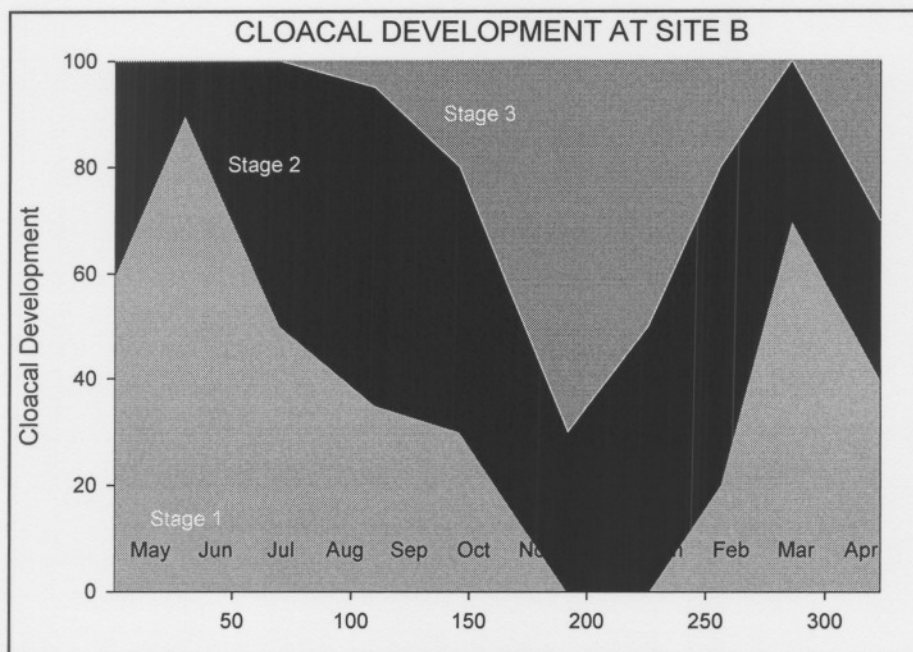
(C)
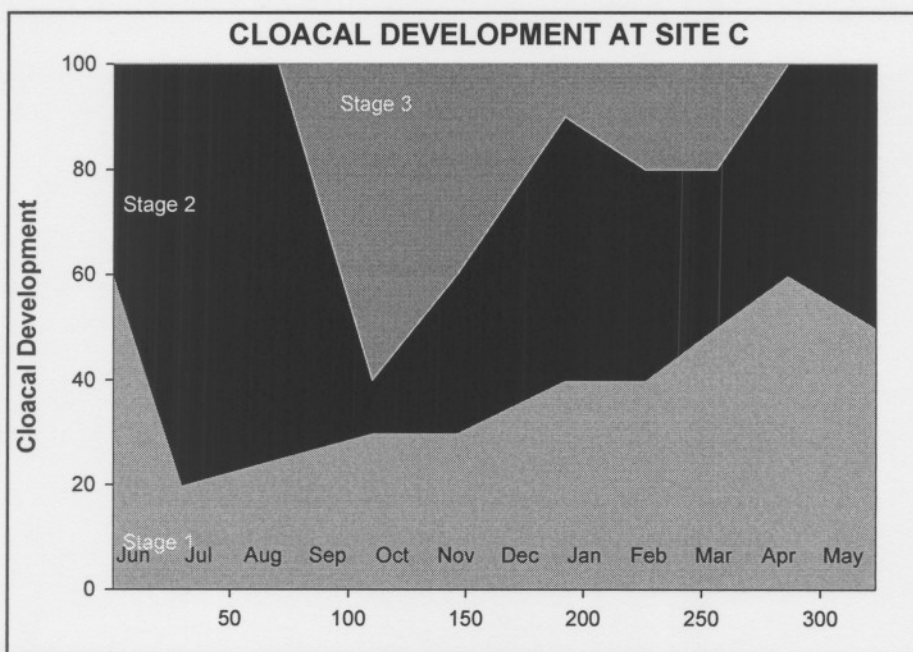
Figure 3.25: Graphs showing the variation in cloacal fold development at (A) Site A (dry period indicated with white block), (B) Site B and (C) Site C during the study period.

3.2.8 Gonadal Anomalies

Gross testicular anomalies observed during the study included discontinued testes, absent testes and testes with one large testis and one small testis (Table 3.5). The prevalence of testicular anomalies at Site A was 3.57%, at Site B 3.84% and at Site C 2.12% as shown in Figure 3.23 and examples in Figure 3.24. Total prevalence = 3.19%.

Table 3.5: Table showing the different types of gross testicular anomalies observed.

GONADAL ANOMALIES			
Site	Discon-tinued (n)	Testis absent (n)	Giant and miniature (n)
A	2	-	1
B	3	1	-
C	2	-	-
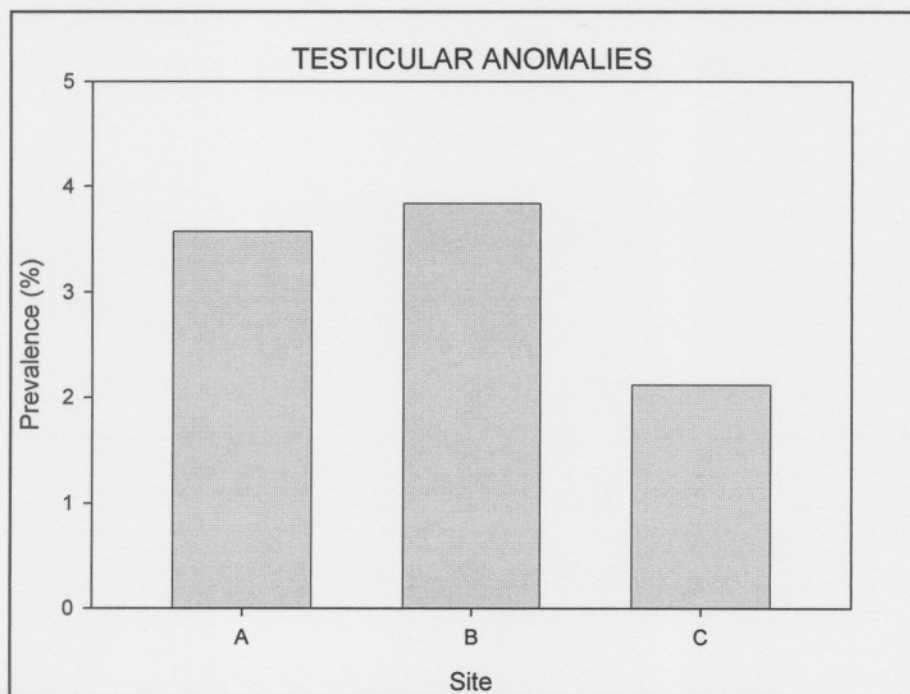
Figure 3.26: Graph showing the prevalence of gross testicular anomalies at the three study sites.

(A)
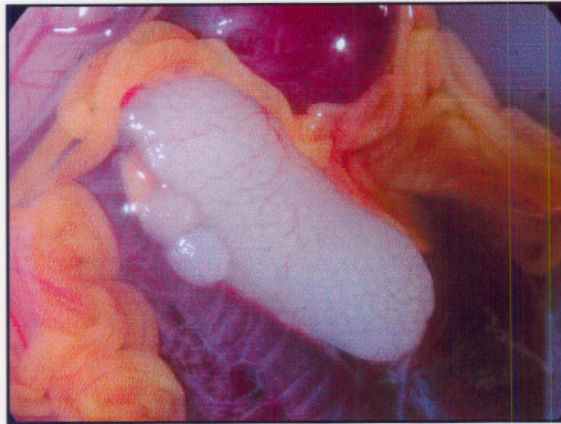
(B)

(C)
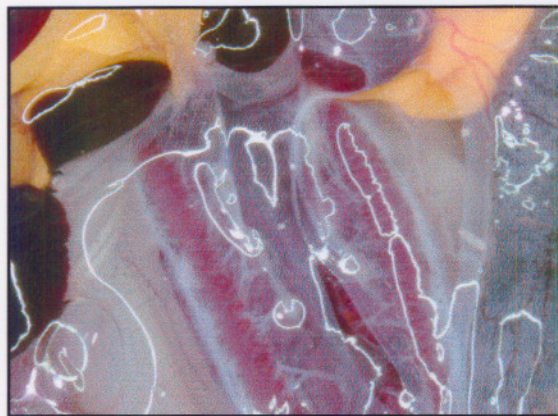
Figure 3.27: Photographs showing the different types of testicular anomalies that occurred during the study period at the three study sites; (A) shows small and discontinued testes, (B) shows discontinued testes and (C) shows absent gonads.

3.3 HISTOMETRICAL ANALYSIS

3.3.1 Testicular Cellular Fractional Volume

The fractional volume (%) of the reproductive cells, namely spermatogonia (Figure 3.28), spermatocytes (Figure 3.29), spermatids (Figure 3.30) and sperm (Figure 3.31) was determined. An increase in fractional volume of spermatogonia was observed for October 2003 followed by a decrease towards November 2003 followed again by an increase towards January 2004 and a decrease towards February / March 2004. The spermatocytes showed a small increase during September 2003 and a decrease thereafter. An increase was again observed towards February / March 2004 with a decrease thereafter. For spermatids, the same phenomenon was observed. Site A showed an increase in sperm production towards November 2003, while site B increased towards September 2003 and site C towards October 2003. All three sites showed a decrease in sperm production thereafter. Site B, however, showed an increase in sperm production towards March 2004 and site C to a lesser extent.
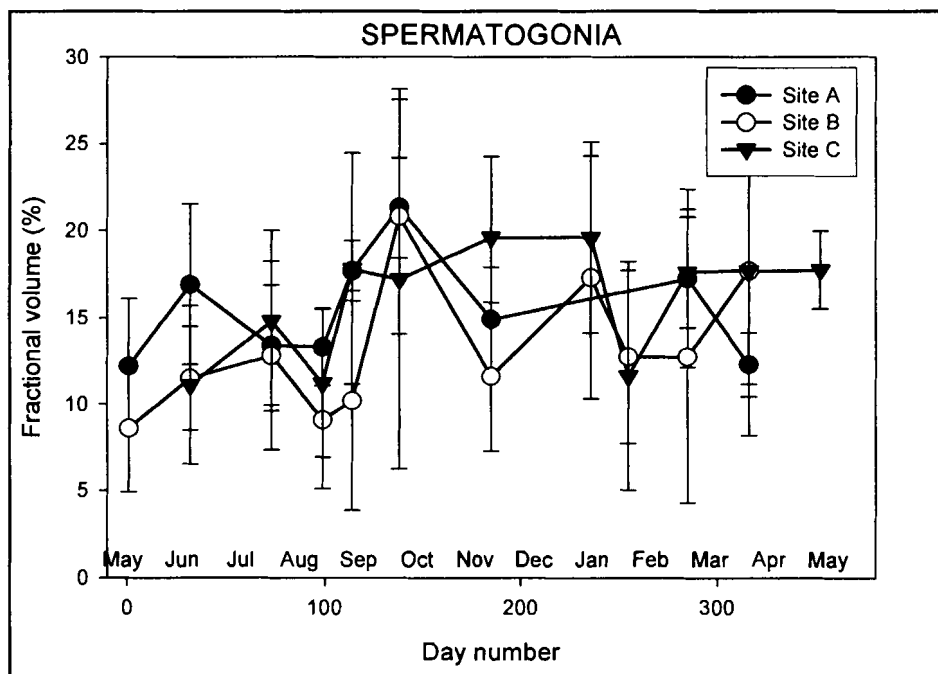
Figure 3.28: Graph showing the fractional volume (%) of the spermatogonia at the three study sites during the study period from May 2003.
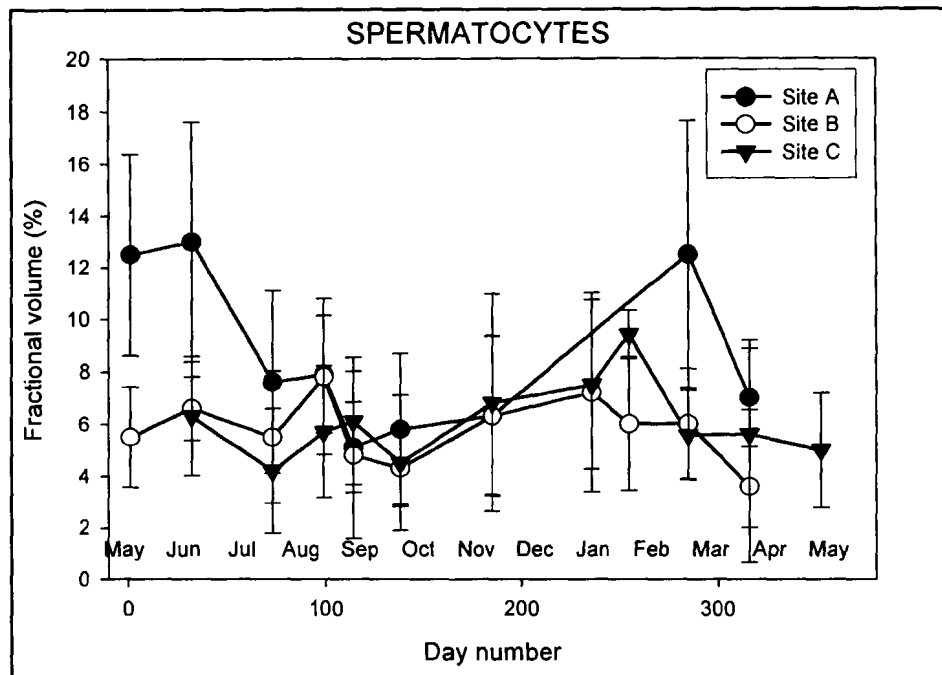
Figure 3.29: Graph showing the fractional volume (%) of the spermatocytes at the three study sites during the study period from May 2003.
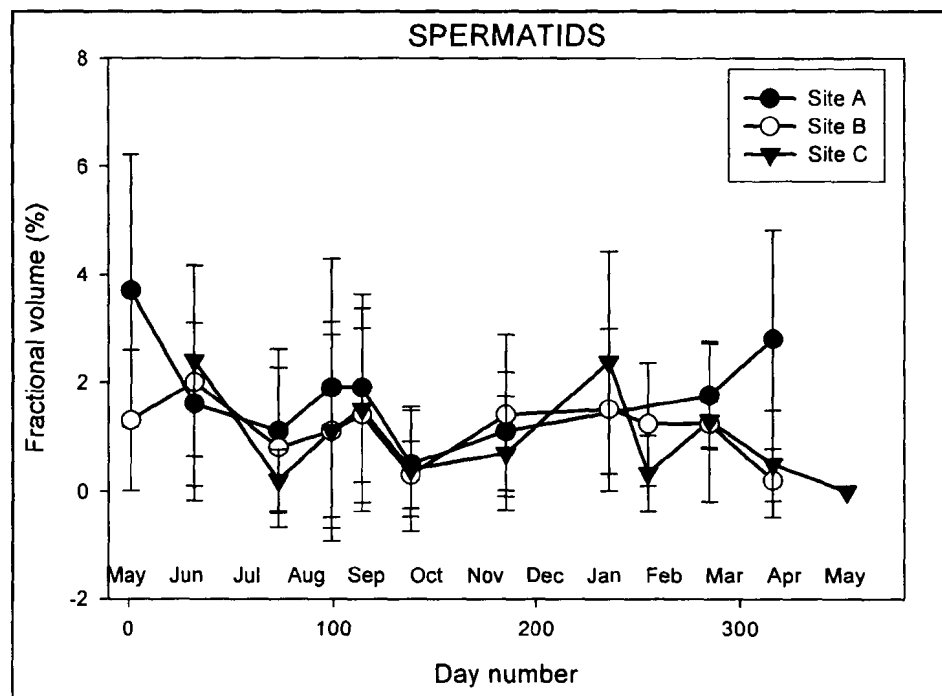
Figure 3.30: Graph showing the fractional volume (%) of the spermatids at the three study sites during the study period from May 2003.
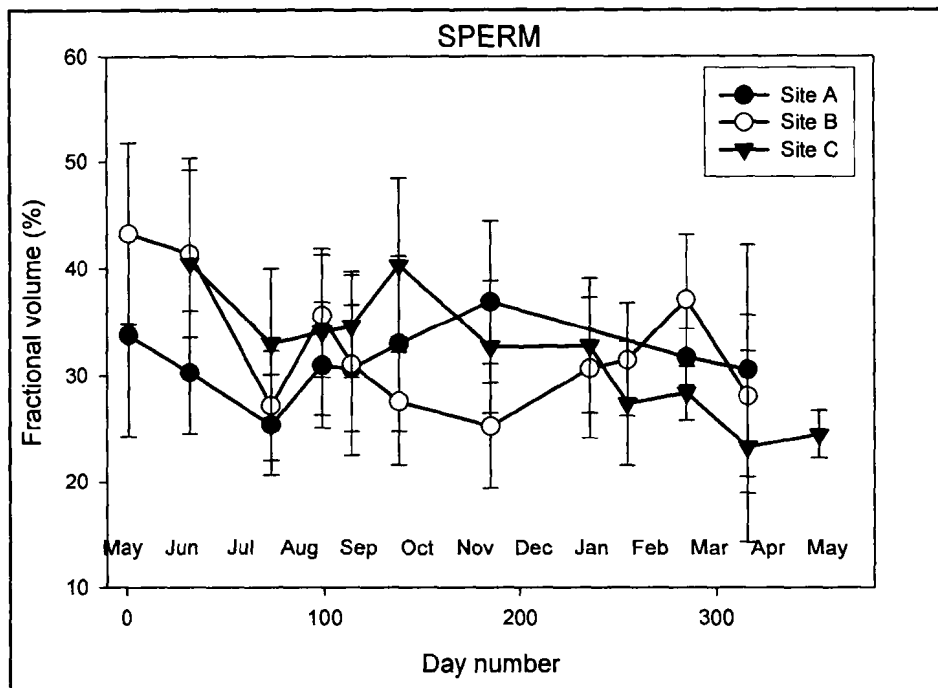
Figure 3.31: Graph showing the fractional volume (%) of the sperm at the three study sites during the study period from May 2003.

3.3.2 Testicular Oocytes

Site C (20.2%) shows the highest prevalence of testicular oocytes among all individuals collected (Figure 3.32), followed by site A (13.1%), with the lowest prevalence at site B (12.5%). The results are given in Table 3.6. The greatest number of oocytes per individual (Figure 3.33) was seen at site B with 20.1%. Site C had 18.2% oocytes per individual and site A had 9.1% oocytes per individual. Two different types of oocytes were observed, namely fully-formed oocytes and smaller regressed oocytes (Figure 3.35). Figure 3.34 shows the specific categories of oocyte prevalence. The highest number off oocytes per individual was 94 regressed oocytes at study site C and 93 regressed oocytes at site B. One specimen at site C had 26 mature oocytes, while another at site B had 16 mature oocytes.

Table 3.6: Table showing the occurrence of testicular oocytes at the three study sites, including the totals of the three sites, during the study period.

Site	A	B	C	Total
Number of male frogs	84	104	94	282
Number of individuals with testicular oocytes	11	13	19	43
Number of mature oocytes	12	38	63	113
Number of regressed oocytes	88	178	282	548
Total number of oocytes	100	261	345	706
Mean number of oocytes per individual	9.1	20.1	18.2	16.4
Prevalence (%)	13.1	12.50	20.2	15.2
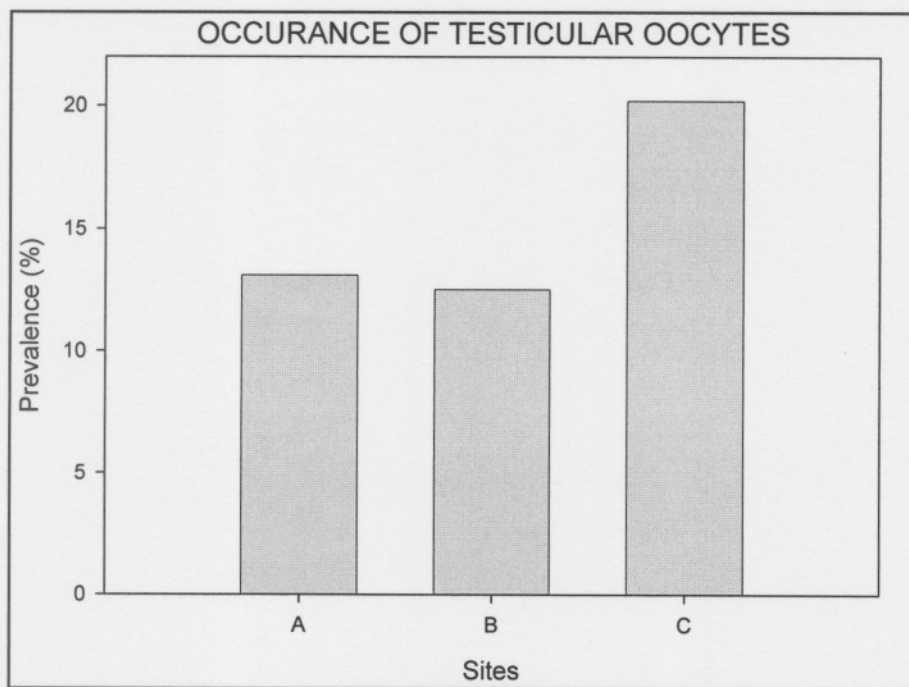
Figure 3.32: Graph showing the prevalence of testicular oocytes at the three study sites.
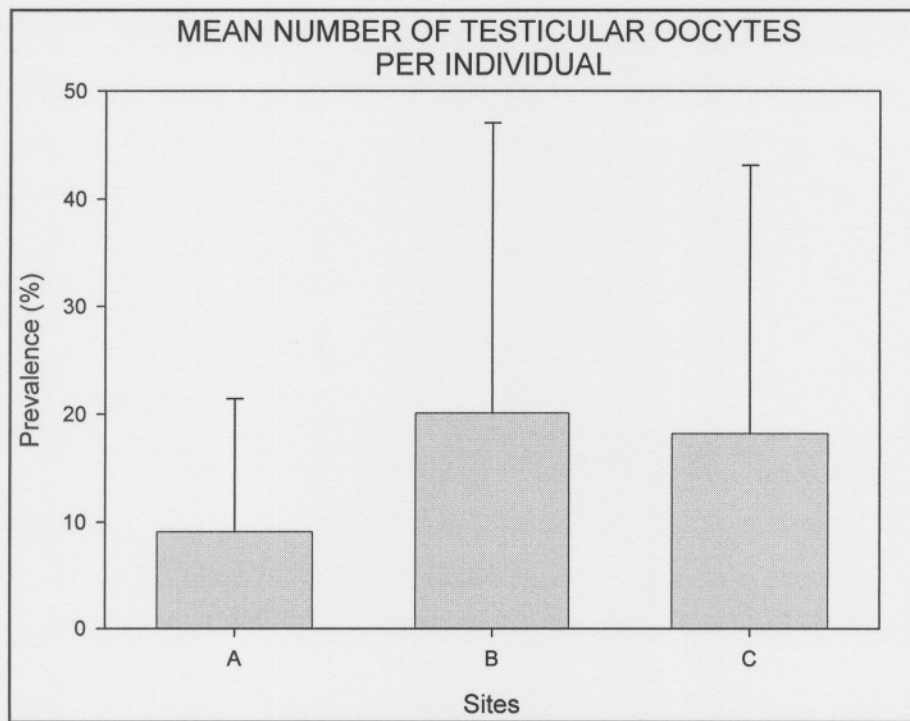
Figure 3.33: Graph showing the mean number of testicular oocytes per individual and the standard deviation at the three study sites.
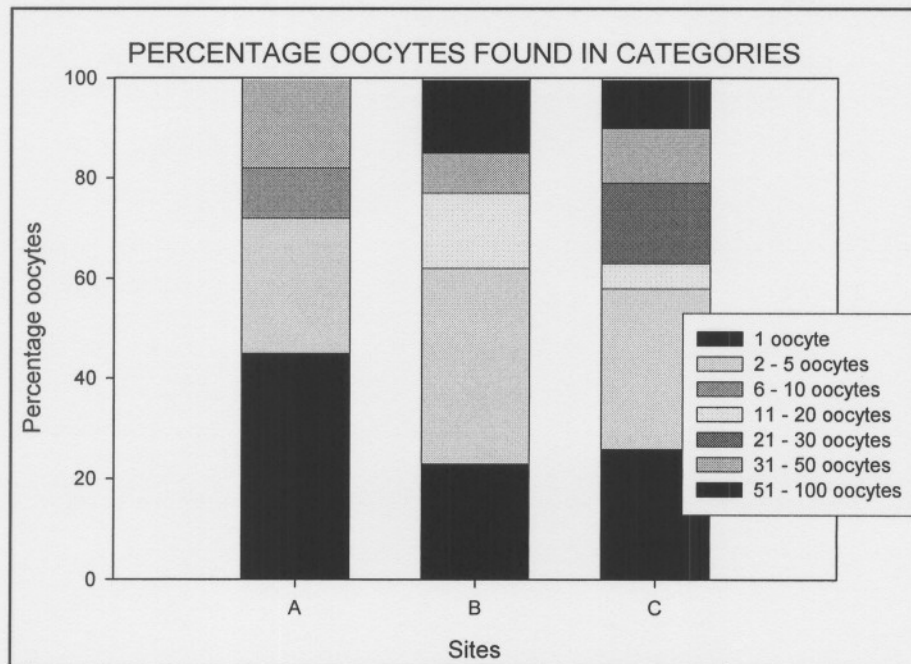
Figure 3.34: Percentage oocytes found in one of the categories specified.
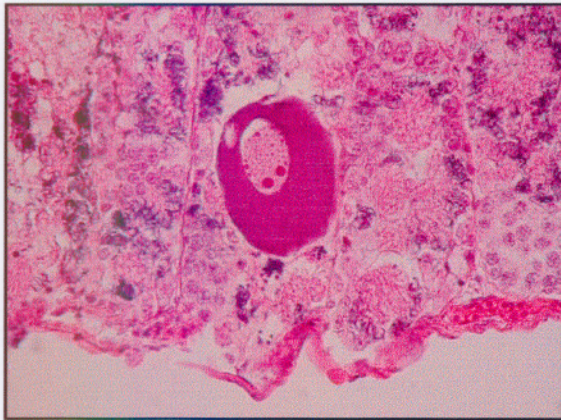
MATURE OOCYTES   **REGRESSED OOCYTES**
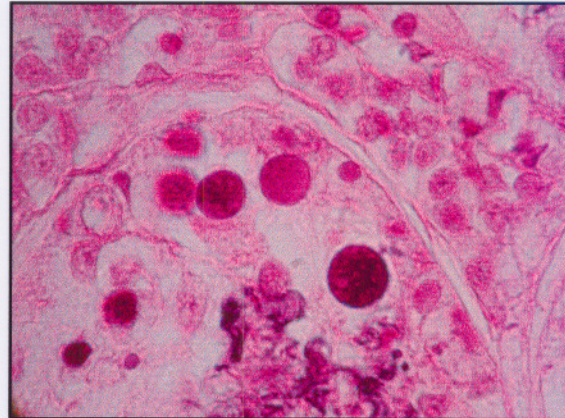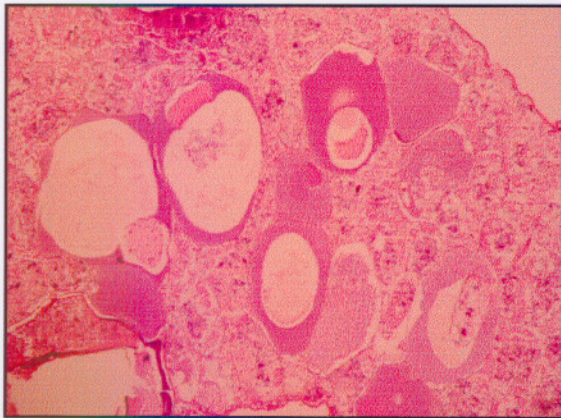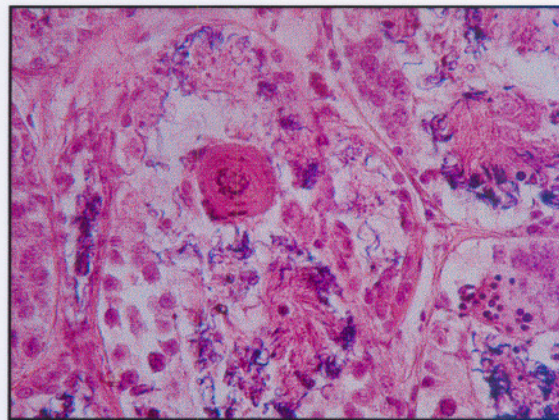
Figure 3.35: Photomicrographs showing the differences between mature and regressed oocytes.

3.3.3 Age Profile

An example of a histological section through the toe of a two-year-old frog is given in Figure 3.36 and a six-year-old frog in Figure 3.37. The age structure of the frogs collected ranged from one to nine years, with the majority of the frogs being two to three years old (Figure 3.38). The mean age profile of all the sites ranged from 2.6 to 3.5 years (Table 3.7). The age structure of frogs with testicular oocytes is given in Table 3.8 and Figure 3.40. The age structure of frogs with testicular oocytes ranged from one to five years old. The mean age profile of all the sites ranged from 2.3 to 2.7 years. The age structure is also given in Figure 3.40 and then evaluating the percentage of frogs with testicular oocytes in each of the age groups in Figure 3.41.
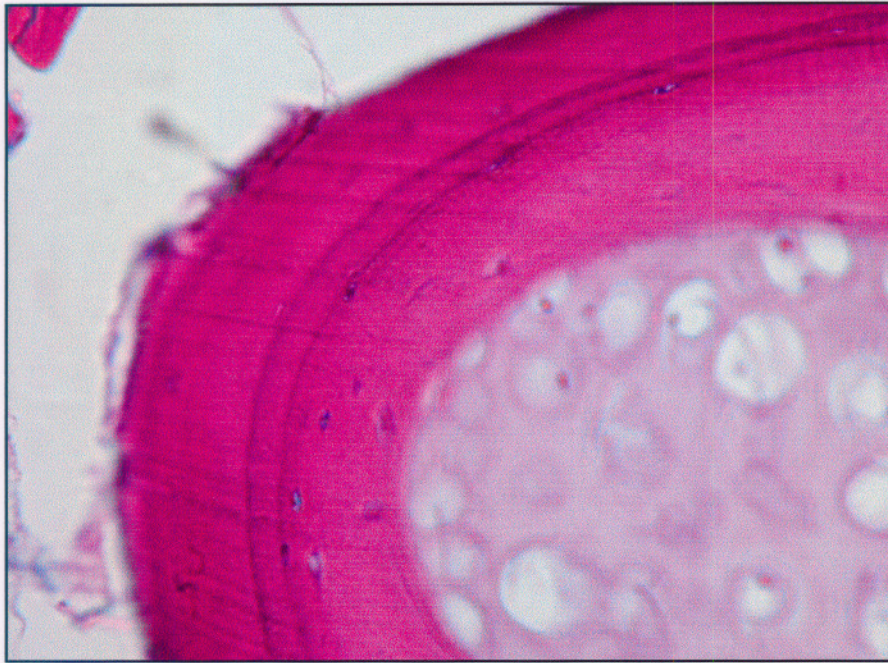
Figure 3.36: Photomicrograph of a histological section through the toe of a 3-year-old frog.
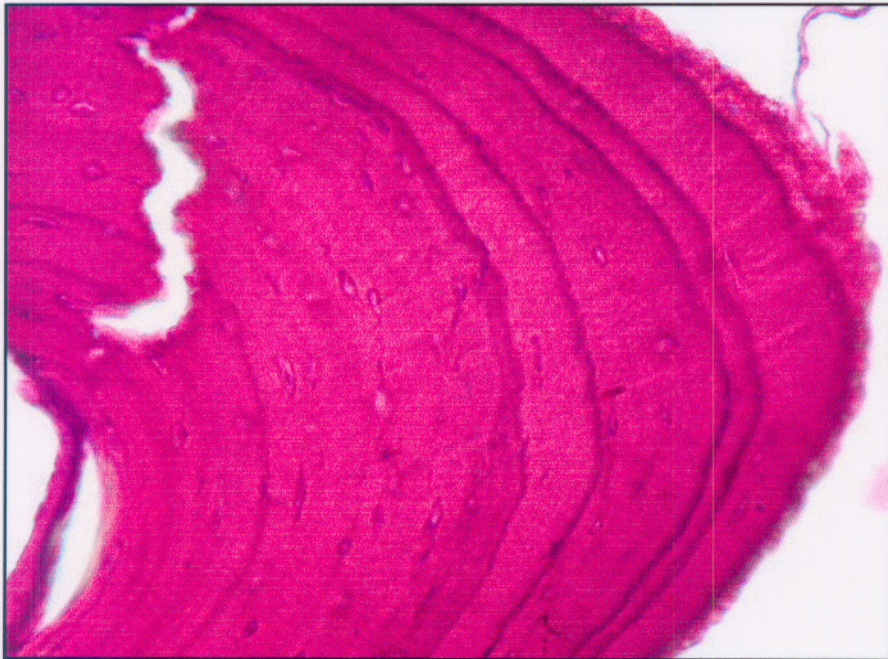
Figure 3.37: Photomicrograph of histological section through the toe of a six-year-old frog.

Table 3.7: Table showing the age structure of the frogs collected at the study sites.

SITE	A	B	C
1 year	7	21	24
2 years	29	34	32
3 years	25	23	20
4 years	15	12	13
5 years	8	4	4
6 years	6	2	3
7 years	4	2	2
8 years	4	2	0
9 years	2	0	2
TOTAL NUMBER	169	214	199
MEAN AGE	3.5	2.6	2.6
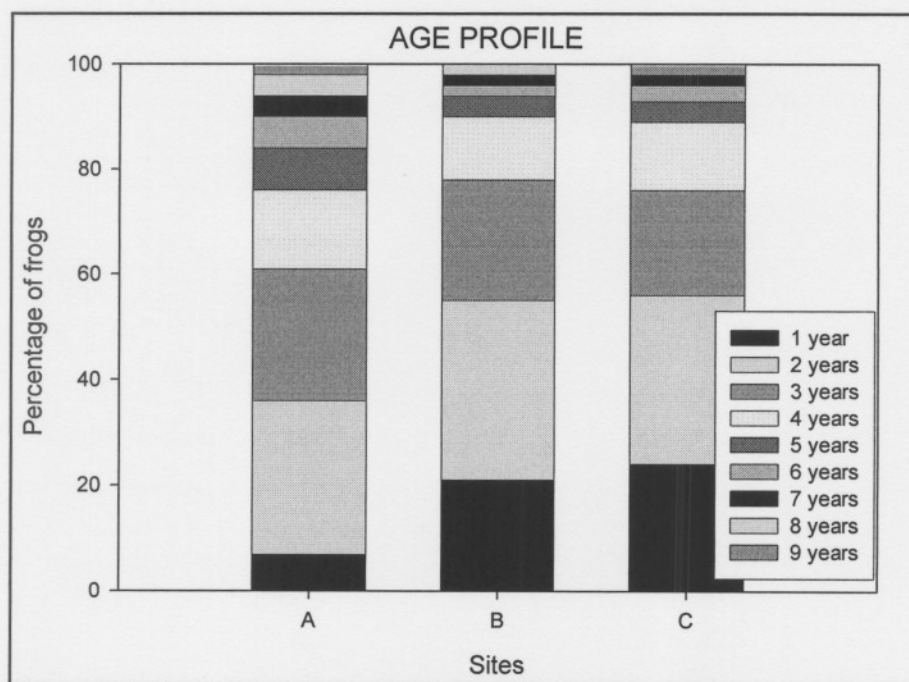
Figure 3.38: Histogram showing the age profile of *Xenopus laevis* collected at the three study sites.

Table 3.8: Table showing the age structure of frogs with testicular oocytes.

	MATURE OOCYTES			REGRESSED OOCYTES		
SITE	A	B	C	A	B	C
1 year	0	2	5	0	1	0
2 years	4	4	2	1	1	2
3 years	2	2	5	2	1	2
4 years	0	1	1	0	0	2
5 years	1	0	0	0	1	0
TOTAL NUMBER	7	9	13	3	4	6
MEAN AGE	2.7	2.2	2.1	2.6	2.7	3
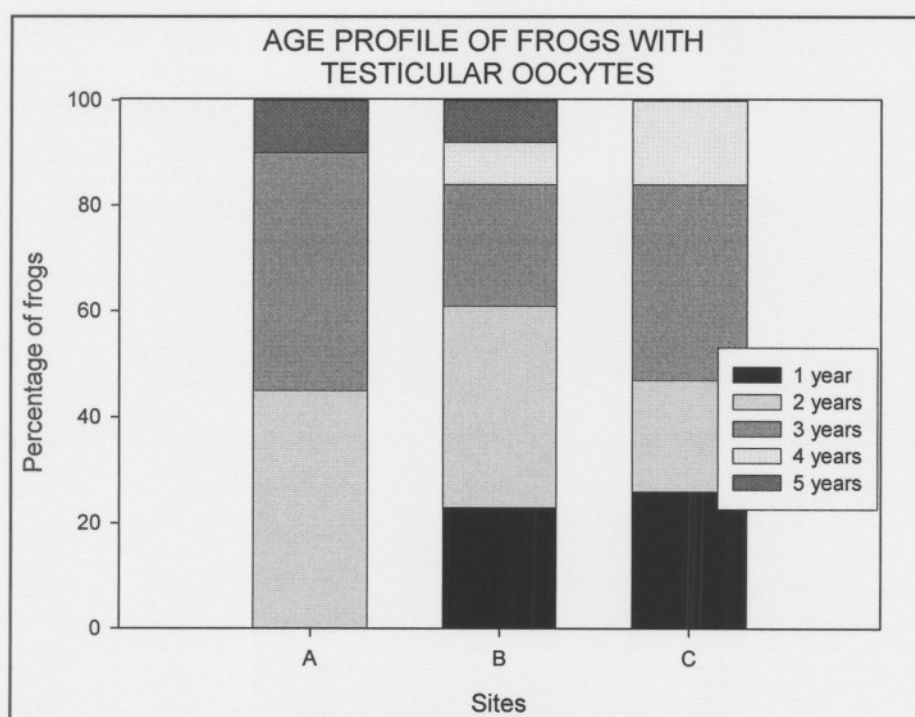
Figure 3.39: Histogram showing the age profile of *Xenopus laevis* with testicular oocytes collected at the three study sites.
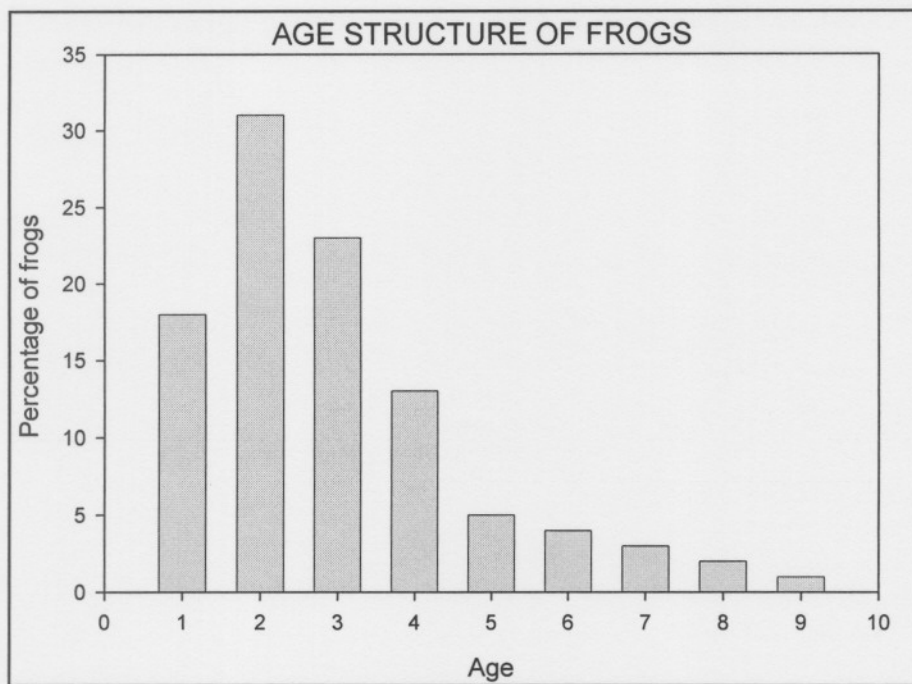
Figure 3.40: Histogram showing the percentage of frogs in each age group.
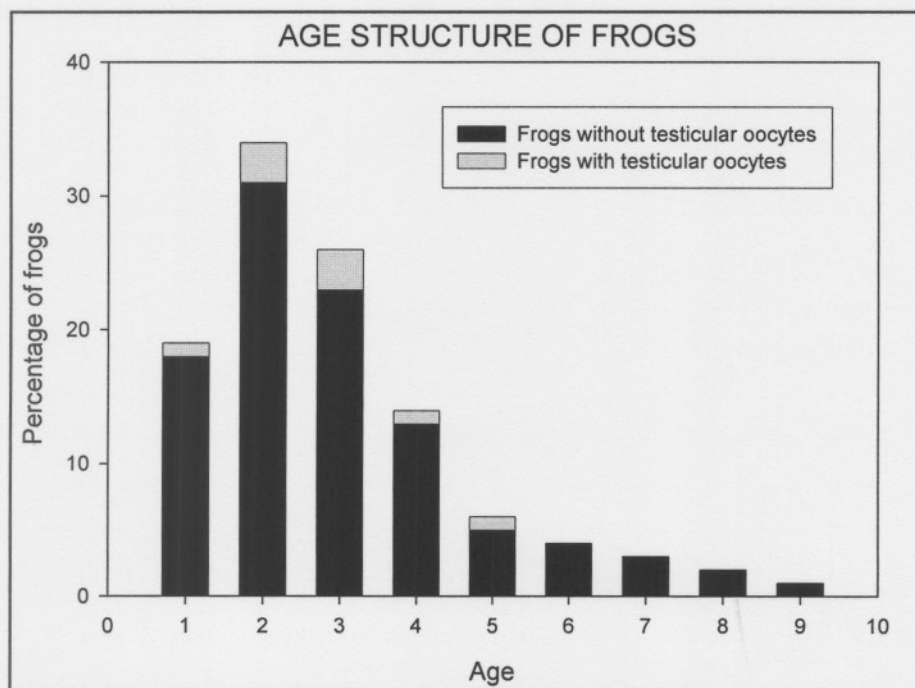
Figure 3.41: Histogram showing the percentage of frogs in each age group with testicular oocytes.

3.4 HORMONAL ANALYSIS

Concentrations (pg/ml) testosterone and oestrogen in female frogs are given in Figure 3.42 (A) and (B). Significant variation between the three study sites was evident during the onset of the breeding season. Concentrations of testosterone and oestrogen increased in the females up to November 2003 and decreased thereafter. Small increases in oestrogen concentrations were observed for March 2004. The concentrations of male testosterone and oestrogen are shown in Figure 3.43 (A) and (B). Again, there were large variations during the start of the breeding season with concentrations increasing towards November 2003 and decreasing thereafter. There was also a slight increase in concentrations at the end of the season.

(A)

(B)
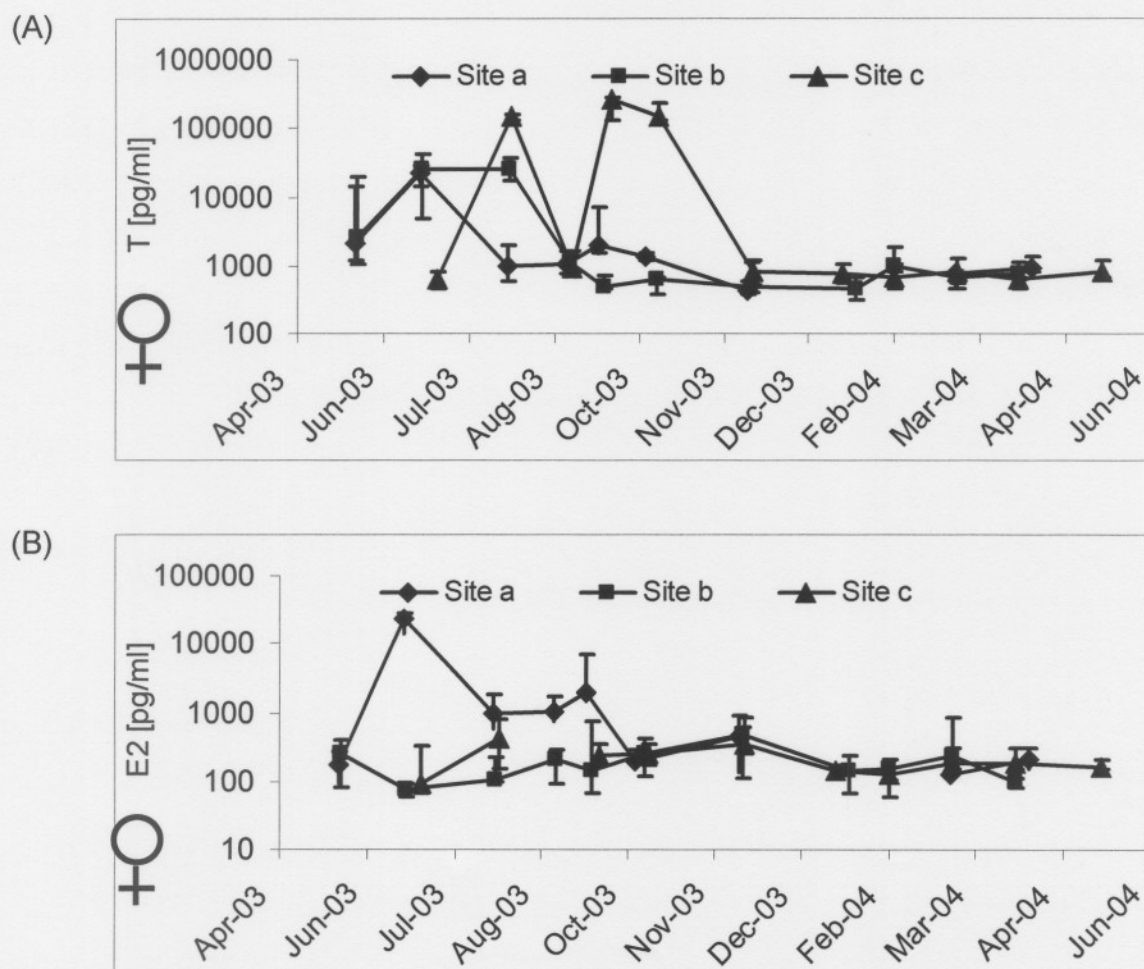
Figure 3.42: Graphs showing the testosterone and oestradiol concentrations, with standard errors, in female frogs from the three sites.

(A)

(B)
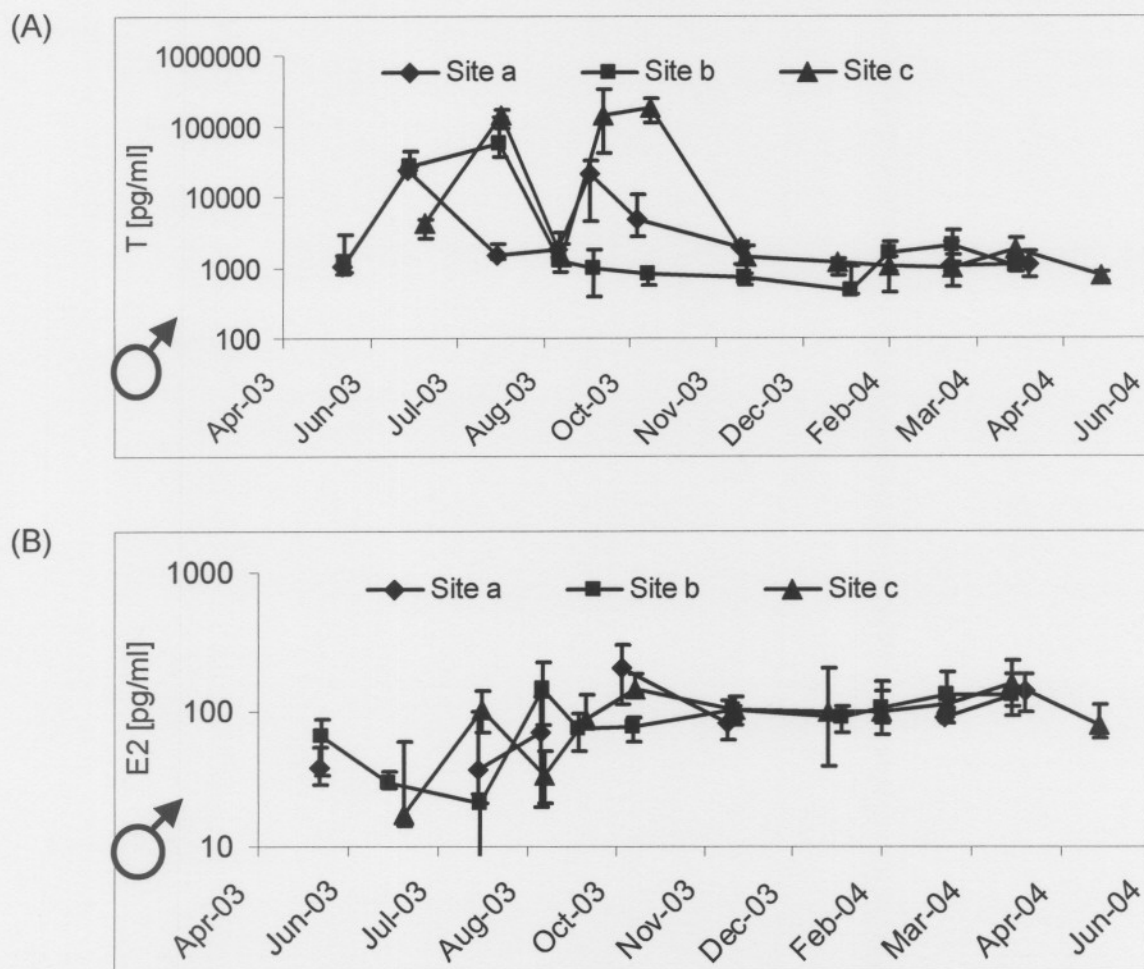
Figure 3.43: Graphs showing the testosterone and oestrogen concentrations, with standard errors, in male frogs from the three sites.

3.5 ECOLOGICAL ASPECTS OF *XENOPUS* REPRODUCTION: A CASE STUDY

3.5.1 Mark and Recapture

The number of animals collected varied significantly between trapping events. The highest number of individuals caught was 44 frogs during November and the smallest number was a single specimen during January. Only 14% of the total sample was males. Based on the statistical calculations using the Jolly-Seber Stochastic Method the estimated population size of site D was 220 (Table 3.9). Population size, population

growth and survival rate were also determined (Table 3.10). The population density was determined to be 0,22 frogs/m^3.

Table 3.9: The results of the mark and recapture of frogs.

i	n_i	r_i	Day of marking (j)
			1	2	3	4	5	6	7	8	9	10	11	12
1	17	17	Recaptures (m_{ij})
2	18	18	10											
3	9	9	6	3										
4	44	44	10	4	0									
5	3	1	1	0	0	1								
6	7	7	3	1	0	3	0							
7	2	2	1	1	0	0	0	0						
8	16	15	3	1	0	5	0	0	0					
9	20	20	1	0	0	5	0	0	0	0				
10	10	10	0	0	0	0	0	0	0	0	0			
11	23	23	0	0	0	6	0	0	0	1	4	2		
12	7	7	0	0	0	1	0	0	0	0	0	2	2	

Table 3.10: Table showing the estimated values of population size (Ni), population growth ($\hat{g}_i$) and survival rate (ϕ_i) for each of the captures.

I	N_i (STA)	ϕ_i	$\hat{g}_i$
1	-	3.24	95
2	95	0.51	-16.05
3	32.4	1.21	78.1
4	117.3	0	0
5	-	-	-
6	-	-	-
7	-	-	-
8	346.8	0.22	61.7
9	138	0.5	-69
10	330	1.19	81.4
11	81.4	-	-
12	-	-	-

3.5.2 Ecological Aspects

Study site D was divided into northern and southern halves as well as in eastern and western halves and habitat preferences compared based on the number of individuals captured in each of the quadrants. The results are given in Figure 3.44 and Figure 3.45. There were no meaningful differences according to the Tukey's Honest-test which gave a p-value higher than 0,05 (Figure 3.44 = 0,143 and Figure 3.45 = 0,635). The northern part of the pond had an average of 13 individuals, while the southern part of the pond had an average of 24 individuals. The p-test showed no meaningful differences, because of the large standard deviations. The western part showed an average of 20 individuals and the eastern part an average of 16 individuals. The p-test showed no statistically-meaningful differences between the western and eastern part and that showed that more frogs preferred the south western region of the pond.
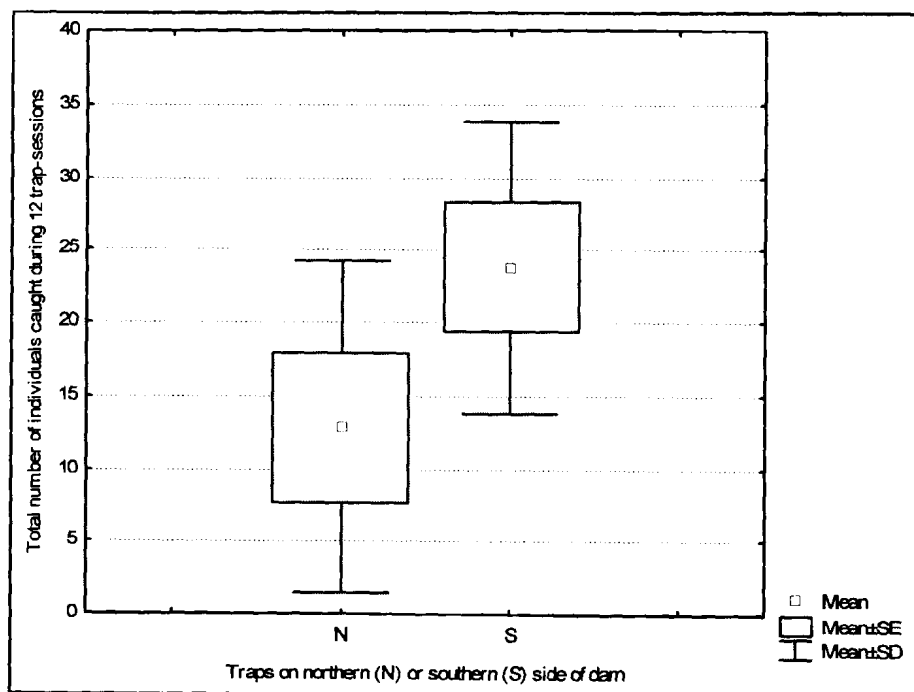
Figure 3.44: Graphs showing the differences in the number of *Xenopus laevis* trapped between the northern and southern parts of the pond.
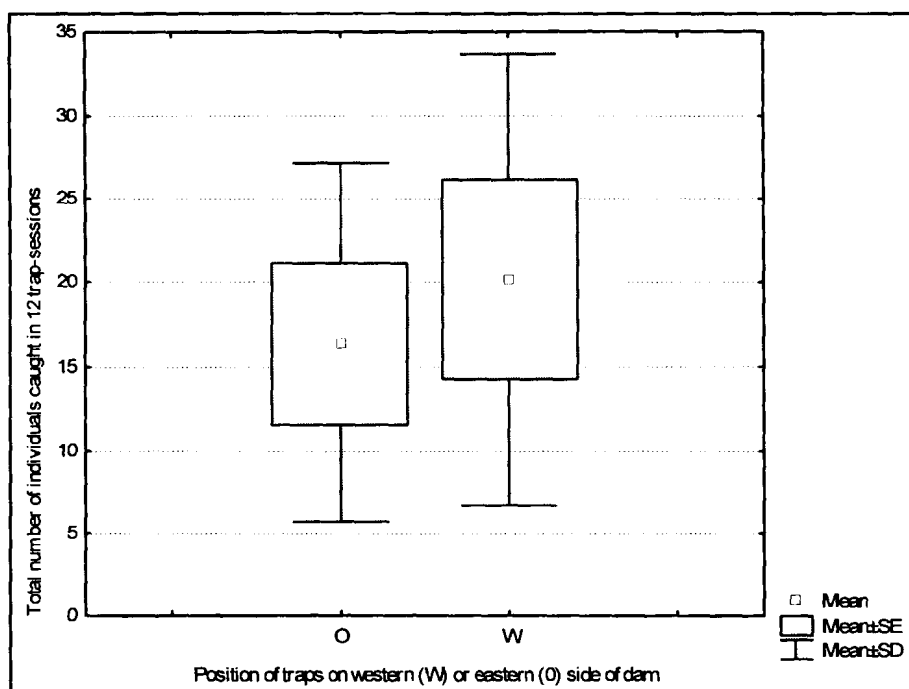
Figure 3.45: Graphs showing the differences in the number of *Xenopus laevis* trapped between the western and southern parts of the pond.

3.5.3 Tadpoles

Xenopus laevis tadpoles were caught on 25 November 2003. A 500 x 500 mm frame was placed in the water at each reference point and all tadpoles inside were caught. The tadpoles were staged using the normal table from Nieuwkoop and Faber (1956). The average length of the tadpoles was 35,8 mm with the most falling in stages 47 to 58. *Cacosternum boettgeri* and *Bufo sp.* tadpoles were also caught. This coincides with the breeding season for *X. laevis* and with the first rainfall of the season.

3.5.4 Other Species

At site D, neither *Xenopus laevis,* nor other species of amphibia were ever found in amplexus. *Bufo gutturalis, Tomopterna cryptotis* and *Kassina senegalensis* could sometimes be heard calling at other nearby dams or ponds. Site D was situated approximately 120 meters from the large Potchefstroom Dam. A setback was the release of water from the site by the owner on two occasions to clean the site. The *X. laevis* could then escape to the big dam, however, this was not noted.

At site B, *Bufo gutturalis* was found one rainy morning in amplexus (Figure 3.46). Soft rain fell the whole morning of 2 January 2004 and about 54 pairs of *B. gutturalis* were counted at any time from 8 am until about 12 am. The shallow water was covered with eggs (Figure 3.47), which were being consumed by crabs. Frogs apparently killed by predators were also visible (Figure 3.48) due to predation, but the predators were not known.
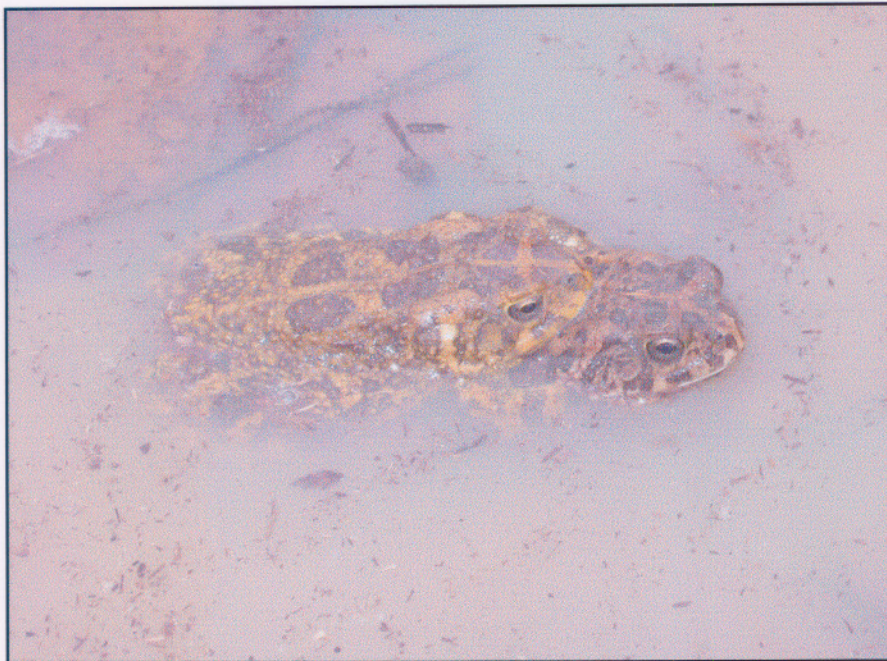
Figure 3.46: Photograph of *Bufo gutturalis* in amplexus.
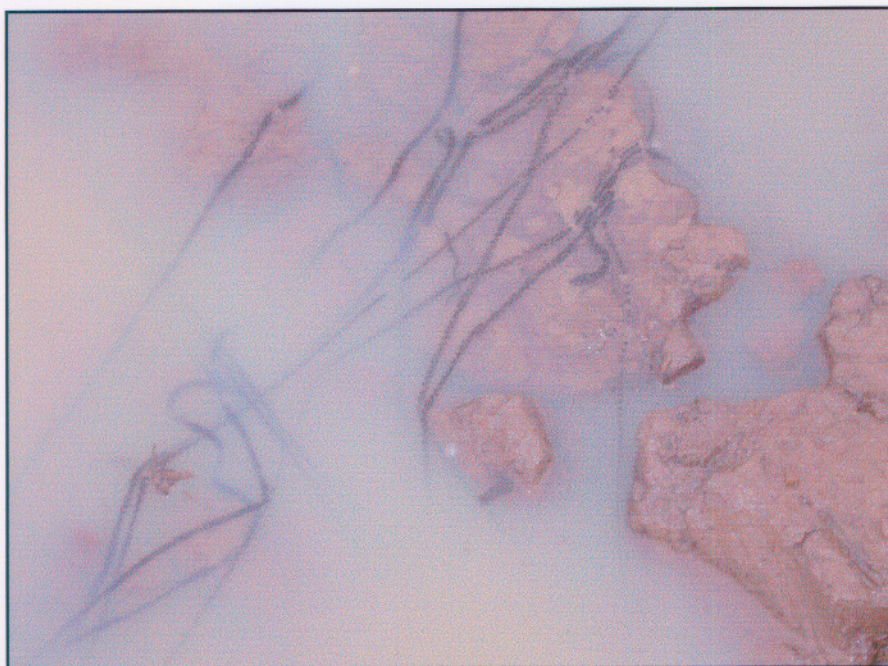
Figure 3.47: Photograph of the eggs from *Bufo gutturalis*.

Figure 3.48: Evidence of predation on *Bufo gutturalis* during reproduction.

CHAPTER 4

DISCUSSION

The aim of the present study was to study the breeding biology of *Xenopus laevis* populations under natural conditions through a period of one calendar year, during which the climatological conditions were hoped to be typical, close to the recorded 10-year mean. The study period, however, turned out to be one of extremes with exceptionally dry and wet periods. The dry periods led to the drying up of one of the study sites (site A) and the water levels to drop drastically at another (site B) during October 2003 and between November 2003 and January 2004. This, however, provided us with the opportunity to see how clawed frogs would perform during very dry periods.

Different ideas exist about the breeding season of the clawed frog and factors influencing it. Balinsky (1969) states that the extended breeding season ranges from the beginning of September to middle of March, while Brown (1970) suggests that it may be from the beginning of August to December and Wood (1965) and Tinsley *et al.* (1996) indicate a biannual breeding during the raining season in Kenya. It is also widely speculated as to what the stimuli may be for inducing breeding. Heyer *et al.* (1994) and Tinsley *et al.* (1996) suggest that breeding is determined by environmental conditions during the days preceding spawning. Brown (1970) states that breeding is controlled by temperature. Bles (1905), Berk (1938), Brown (1970) and Savage (1971) report that when the mid-afternoon temperature of the water rises above 21°C, spawning would be abundant on the following day. The other main factor may be rainfall. Wood (1965) and Tinsley (1996) argue that most records show that the breeding season coincides with the start of the rainy season. Heavy rain would wash fresh sediments into the ponds and, as a result, enrich the pond with nutrients and promote algal and phytoplankton blooms. Bles (1905) and Tinsley *et al.* (1996) state that the production of oocytes is determined by food supply and that the ovaries regress during food shortage and overcrowding.

The present study showed that the preparations for reproduction started during August 2003 and continued up to March 2004. The results will also be discussed later in this chapter. It was also noticeable that breeding coincided with the major rainfall events. It is thus true that the breeding season extends from August to March, but that breeding is mainly influenced by rainfall, the availability of food and temperature variations as a result of that. This also influenced the site that dried up (site A). It is evident from the results that breeding took place much later at this site. The air and water temperature started increasing from August and decreased during March, which again coincided with the breeding season. The pH at each of the study sites was found to be in the range described in the literature. Nieukoop and Faber (1956), Visser (1962) and Tinsley (1973) stated that the pH varied between 5.6 and 8.7 for X. laevis habitats. Dissolved oxygen showed low values from August to March. This indicates an increase in eutrophication from increases in alga in the ponds during the breeding season. This may also coincide with an increase in the plankton community and bacterial activity. This supports the theory that breeding is initiated during times of increased availability of food for the tadpoles.

During the study, 282 adult male and 300 adult female X. laevis were collected from three study sites. McCoid and Fritts (1989) recorded that male frogs become sexually mature at age 12 months and the females at age 15 months. The mass of the frogs varied greatly during the breeding season. That may be due to the changes in the fat bodies (Gitlin, 1941) and the continuous process of ovulation (Dumont, 1972 and Weisman, 1944).

There was large variation in several morphological measures of frogs from the three study sites. These include mean snout-vent length and mean mass of frogs from the three sites. Each site must be considered individually and different sites in a specific area must be taken as individual entities. The gross morphology of the gonads also shows an increase in the mean mass with the onset of the first rains and a decrease thereafter. The mean mass of the gonads then increased again during the onset of the second major rainfall and decreased after that. The gonado-somatic index (GSI), which

is the percentage of the gonads of the total body mass off the frog, also showed an increase during the onset of the breeding season in August and then decreased after the first rain. The GSI then increased again with the onset of the second rainy period. The theory of continuous ovulation is supported with this study. Mature ova (stage 3) were present throughout the study period at all three study sites, but peaked during October and November. Gitlin (1941) reported that fat bodies might reach maximum mass during aestivation and minimum mass during the breeding season. Dumont (1972) and Weisman (1944) found that oogenesis in *X. laevis* is a continuous process and that ovulation may occur on a yearlong basis. Gitlin (1941) also found that when females continue to grow, the number of eggs ovulated increased with body size.

Nuptial pads started to develop in male frogs from July and peaked during November, where after they disappeared during March. This coincides with the breeding season of *X. laevis*. This is also true for the cloacal development in the females. Again, maximum development (stage 3) was observed between October and February. The testicular cellular fractional volume of the reproductive cells also supports this observation. Reproductive cells started to increase during August up to the first rainfall, where after it decreased, but increased again up to the second rainfall. The presence of sperm cells was seen throughout the study period at the three study sites. This indicates that mature ova and sperm are always present and that the male and female *X. laevis* are ready to breed at any time when conditions are favourable.

The development of *X. laevis* is divided into 66 stages from fertilisation to completion of metamorphosis (Nieuwkoop & Faber, 1956). The developing gonads can first be recognised at stage 46 as thickenings on both sides of the dorsal root of the dorsal mesentery (Iwasawa & Yamaguchi, 1984). The primordial germ cells are visible from stage 40 (Nieuwkoop & Faber, 1956) and, by stage 49, have migrated into the developing gonad and are enveloped by epithelial cells. The testes cannot be distinguished from the ovaries between stages 46 and 55 and this period is known as "indifferent" (Iwasawa & Yamaguchi, 1984). During the indifferent phase, cells are added to the proximal portion of the gonad and it then enlarges and the interior medulla

can be distinguished from the exterior cortex. In females, a cavity appears in the medulla, which is then transformed to epithelium. The primordial germ cells are located in the cortex. In males, the primordial germ cells migrate into the medulla and no cavity is formed. Between stages 56 and 66, the primordial germ cells, in the female, become surrounded by follicle cells and differentiate into oocytes. In males, seminiferous tubules develop and primordial germ cells differentiate into spermatogonia (Kelley, 1996).

The process of metamorphosis in amphibians has the potential for being sensitive to chemical perturbation of development, because metamorphosis is under hormonal control (McNabb, 1999 and Jooste, 2003). In addition, it is easy to observe and quantify the changes in gross morphology that follows metamorphosis. Many factors can influence gonadal development during tissue differentiation associated with reproduction. These factors may include:

- Hormonal and environmental factors.

- Changes in enzymes that control steroid synthesis and metabolism (Piferrer, 1994).

- Exposure to steroids for long periods and high concentrations (Di Giulio, 1999) at stages outside the critical windows of sex determination and differentiation.

- Environmental chemicals that act as hormone modulators or antagonists.

Hayes (2002) and Tavera-Mendoza (2002), are of the opinion that amphibians exposed to atrazine during embryonic development may undergo feminisation or abnormal gonadal development. They observed what they called, gonadal dysgenesis and hermaphrodites in treated animals. They stated that atrazine might affect sex differentiation by inducing aromatase, the enzyme that converts androgens into oestrogens. This can cause inappropriate synthesis and secretion of oestrogens in males at the expense of androgens. Inhibition of spermatogenesis may be a secondary effect, because of the synthesis of oestrogens in males, rather than a direct effect of the atrazine. After quantitatively assessing testicular volume, primary spermatogonial cell nests, and nursing cells from histological sections, Tavera-Mendoza *et al.* (2002) reported a 57% reduction in testicular volume in tadpoles exposed to atrazine at 18 µg/L

relative to the controls. Similarly, primary spermatogonial cells nests, were 70% fewer than in unexposed individuals while the nursing cells that provide the nutritive support for development of germ cells were 74% fewer (Tavera-Mendoza *et al.* 2002). Unfortunately, the data in the published papers and in the thesis (Tavera-Mendoza, 2001) are inconsistent. For example, the number of animals that were used in the study was not clearly reported and was different in the description of the methods and the figure captions. In the Tavera-Mendoza thesis (2001), it is stated that six tanks were used with 15 tadpoles each, however, in the published paper (Tavera-Mendoza *et al.* 2002) it is stated that two control tanks and two exposed tanks were used, each with 24 tadpoles. In another experiment, more tadpoles were sampled than were initially stated to be present in the tanks. Responses at greater concentrations may have been confounded by the general necrosis observed in several tissues of exposed tadpoles (Tavera-Mendoza, 2001). The cause of the necrosis was not clear. The authors reported that testicular volume was reduced in atrazine-exposed tadpoles, but they did not make measurements of testicular volume in a sub-sample of animals before exposure to atrazine. Furthermore, the responses reported in the thesis were inconsistent with those reported elsewhere (Carr *et al.*, 2003, Hayes *et al.*, 2001 and Hecker *et al.*, 2003). It was also noted that atrazine may not be the only compound that induces testicular oogenesis; however, in conjunction with other products it may cause the phenomenon. Cooper (2000) found that atrazine might interfere with the endocrine regulation of reproduction through the hypothalamus in rats and it has been reported to have effects on gonadal aromatase activity in alligators, however, there were no other effects on reproduction and development (Crain *et al.*, 1997 and 1999).

Contrary to these reports, Jooste *et al.* (2005) suggested that gonadal anomalies and testicular oocytes occur in both atrazine-exposed and control *X. laevis*. They further stated that there were no concentration responses between the frequency of oocytes and the total number of oocytes in individual frogs. Carr *et al.* (2003) failed to demonstrate any effect of atrazine on laryngeal dilator muscle, but at high concentrations (25 µg/L) found small increased frequencies of discontinuous gonads. Coady *et al.* (2005 and 2004) reported that atrazine had no effect on gonadal

development or the frequency of gonadal anomalies in X. laevis and Rana pipiens. Allran (2001) found no effect of atrazine on the metamorphosis or developmental rate of R. pipiens. Furthermore, Smith (2005) found that there was no difference in the fractional volume of the testicular cell types between X. laevis from maize growing areas and non-maize growing areas. Gallien (1974) also described the presence of oocytes in young male testes, because of a juvenile intersexuality phase. This would indicate that the presence of testicular oocytes is a natural phenomenon.

Traces of atrazine were detected at two of the three sites. The concentration at site A was 0.11 µg/L, site B was ≤ 0.01 µg/L and at site C, it was 2.13 µg/L. When the results from the gonadal anomalies were evaluated, it was found that there was no correlation with the concentrations of atrazine at the three sites. Site B, which had no atrazine detected, showed 3.8% of frogs with gonadal anomalies. Site A showed 3.6% with gonadal anomalies and site C, which showed the highest concentration of atrazine detected, had a prevalence of 2.1% of frogs at site C. When assessing the presence of testicular oocytes, no clear relationship was evident. Although site B had the lowest prevalence of testicular oocytes (12.5% of individuals), it had the greatest number of oocytes per individual with a mean of 20.1. Frogs at site A had the second highest prevalence (13.1% of individuals), but a mean of 9.1 testicular oocytes per individual. The prevalence in frogs at site C was 20.2% of individuals with a mean of 18.2 testicular oocytes per individual. The presence of smaller, more regressive testicular oocytes was noted. They had all the characteristics of the adult oocytes, but were much smaller. It is possible that in adult frogs, oocytes are absorbed by the testes and that the regressed oocytes are the remnants of this process. This is consistent with the observed decreased in incidence and number of oocytes in older male frogs (Figure 3.41).

Reproductive hormones also play an important role in amphibian breeding. It is important to understand what endocrine glands are and what function they have in X. laevis. Chemical substances set free in the circulation, namely hormones, may bring about the coordination of activities of the various organs of the body. The effects of the products of endocrine glands control many types of form and function and range, from

influencing the rate of development, the coloration of the skin and the growth of the secondary sexual characters to the appearance of various types of behaviour. Urodele larvae are frequently found sexually mature in nature, showing that the development of the gonads is not dependent on the thyroid hormone. The *pars anterior* of the pituitary have a specific effect on the growth and liberation of the sex products of the adult *X. laevis*. Daily transplants of fresh anterior pituitary cause a spring breeder, *Rana pipiens*, to breed out of season. The gonad-stimulating hormone has practical value, for it provides a ready means of obtaining embryological material at any time of the year (Noble, 1954). Steroid hormones restore sexual responses while gonadectomy eliminates sexual responses. Hormone accumulation in the hypothalamus and infundibulum regulates the activity of neural circuits that mediate sexual behaviours. Emerson (2001) also noted that androgen concentrations vary considerably among breeding male frogs. Males of frog species with relatively larger testes had higher plasma androgen levels than males of species with relatively smaller testes. He concluded that while androgens appear to be necessary but not sufficient to induce calling behaviour, androgen levels vary significantly with aspects of vocalisation. Hearing of species-specific vocalisation definitely increases androgen levels in breeding males (Obert, 1977). Kang (1995) gives another function of androgens in *X. laevis*. The secretion of androgenic steroids is required for the development of secondary sexual characteristics. Masculinisation of the *X. laevis* larynx is detected at tadpole stage 62 when the laryngeal muscle in males is greater than in females. This event depends on androgen secretion from the testes. The summer androgen values in males exceeded those of females by a factor of 7 or 8. This may be due to the appearance of male-specific reproductive behaviours, such as clasping and calling during the summer months, which require high levels of circulating androgens.

The gonads, while primarily organs of reproduction, release hormones into the blood stream which have an important function in stimulating the growth and maintaining the development of the secondary sexual characters, for example the nuptial pads. The secondary characters include differences in red cell count, lung size, behaviour patterns, and many other structural and physiological differences between the sexes

(Noble, 1954). It seems that the testis induces and maintains the secondary sexual characters of the male. The stomal cells surrounding the lobules of the testis produce the testicular hormone of amphibians. When the spermatozoa are released from the lobule and the Sertoli cells undergo degeneration, the stomal cells increase by mitosis, change their form, and exhibit lipoidal droplets and fuchsinophil granules in their cytoplasm (Noble, 1954). Nishimura (1997) explains that synthetic compounds and natural chemicals in the environment may have disrupting effects on foetal development and reproductive systems, acting as oestrogens, anti-oestrogens, anti-androgens or thyro-active or anti-thyroid agents. He further states that oestrogens play an important role in adult female reproduction, especially in vitellogenesis in *X. laevis*. Endogenous yolk deposition is observed at 5 to 6 months after metamorphosis, when the oviduct also presents evidence of oestrogen-dependent maturation. In *X. laevis* tadpoles, differentiation of the indifferent gonad into an ovary starts at stage 52, while vitellogenin synthesis (Polzonetti-Magni, 1990) starts at stage 62 and ovarian oestrogens are beginning to be secreted. May (1980) stated that anurans are not capable of a vitellogenic response to oestrogens throughout the lifecycle. Guerriero (2000) found that levels of hypothalamic oestrogen receptors fluctuated in synchrony throughout the reproductive cycle in females. It is low during the pre-breeding period and high during the breeding and recovery periods. In some cases testosterone does play a role in female anura (DiFiore, 1998). It could play a role in its conversion to 17ß-estradiol by aromatase, which is present in frog liver tissue. In *R. esculenta*, testosterone is positively correlated with ovarian weight. Its function is to restore the dorsal epidermis and mucous gland that regressed after gonadectomy. In *R. pipiens*, testosterone induces oviduct growth. In *X. laevis*, however, vitellogenin synthesis by amphibian liver is a specific oestrogen-dependant process. D'Istria (1974) studied male *R. esculenta* and found that the levels of testosterone are consistent with the morphological modifications of secondary sexual characters during the annual reproductive cycle.

The metabolism of amphibians is greatly affected by the secretions from the glands of internal secretion, especially the thyroid hormone. During the breeding season there is a distinct rise of the metabolic rate, which is mostly observed in the males. It is noteworthy

that animals that differentiate early and reproduce at a small size probably never reach the age of the slow-growing, less-differentiated types (Noble, 1954). Secondary sexual differentiation is directed by secretion of steroid hormones. In females, differentiation of secondary sex characteristics is independent of gonadal steroids until late juvenile stages when oestrogen secretion promotes the development of the oviduct and the synthesis of vitellogenin from the liver. Sensitivity to the gonadal steroids develops earlier, during tadpole stage 56, in both sexes and its onset requires thyroxin secretion. Sexual receptivity in adult females is increased by gonadotropins. Oestrogen and progesterone stimulate female receptive behaviours while prostaglandins suppress unreceptive behaviours including the release call, ticking. In males, androgen is secreted during late tadpole stages and initiates a cellular programme for masculinisation in androgen target tissues that include the vocal organ and the central nervous system. In adult males, the gonadotropin-evoked release of spermatozoa is accompanied by increases in circulating androgens required for clasping behaviour and mate-calling (Tinsley and Kobel, 1996). Hormone-behaviour relationships are regulated by proximate cues (Burmeister, 2000). Successful reproductive behaviour depends on appropriate expression of behaviour associated with male-male competition and courtship, as well as the reproduction of gametes. Hutchinson (2002) stresses the need for further research in order to explore the potential of an assay and to develop biochemical or molecular endpoints to provide the necessary specificity to hormonal disrupters.

Aromatase catalyses the conversion of androgens to oestrogens (Miyashita, 2000) and is a rate-limiting step in oestrogen biosynthesis. Oestrogen biosynthesis in the ovary and brain is catalysed through the expression of enzyme aromatase P450 (Tavera-Mendoza, 2002). This enzyme is the product of the CYP19 gene. This gene is a member of a super family of genes containing more than 300 members in some 36 gene families (Nelson, 1993). Aromatase converts androgens to oestrogen by binding the C19 androgen substrate and catalysing several reactions, thus leading to the ring characteristic of oestrogens (Simpson, 1997). Guerriero (2000) states that while aromatase enzyme activity varies during reproduction, the relative levels of aromatase

protein in *Rana esculenta* show little change during the reproductive cycle suggesting that post-translational mechanisms may be involved in regulating oestrogen synthesis in the frog brain. He further states that sex steroids have potent effects on the nervous system and those androgens and oestrogens control gonadotropin release and evoke expression of sexual behaviour. In addition, oestrogens, through the aromatisation of circulating androgens, mediate certain androgen action, such as oestrogen concentration and receptors. Autoradiography and immunocytochemical studies demonstrated, respectively, widespread distribution of androgen and oestrogen-concentrating nerve cells in hypothalamic and limbic structures of *X. laevis* (Kelley, 1975). Khan (1998) found that liver size, liver microsomal protein and P450 concentrations as well as the levels of oxygenase activity keep increasing with increases in body weight during post-larval development and become stabilised when the adult stage is reached.

Stress and reproduction is introduced into this discussion, because an increasing number of studies report positive association between reproduction and corticosterone that contradict the generalisation that stress inhibits reproduction (Moore, 2003). Moderately-elevated levels of stress hormones appear to facilitate reproduction by mobilizing energy stores. Pronounced activation of the hypothalamo-pituitory-adrenocortical (HPA) axis and extremely elevated levels of stress hormones appear to inhibit reproduction. Animals in nature face environmental, social and physical perturbations, which can jeopardise not only their immediate survival but also other key life history factors such as reproduction. A major function of the HPA axis is to behaviourally or physiologically eliminate or reduce the impact of potential stressors on an animal and thus promote their survival. Physiological effects can include mobilising energy stores, gluconeogenesis and suppressing growth and reproduction (Greenberg, 1987). Behavioural effects can include suppressing reproductive and territorial behaviours and promoting escape behaviours. The HPA axis can function to mediate diverse responses to various stressors that can either increase or reduce the probability of an animal's survival depending on the animal's situation. Stress is thought to have a negative impact on reproduction at multiple levels (Greenberg, 1987). The inhibitory

relationship between stress and reproduction cannot be generalised for at least two reasons (Moore, 2003). First, it appears that, for many animals, the relationship between stress and reproduction in highly context-dependent and same individuals may exhibit an inhibitory response while others do not. Secondly, it is evident that, during reproduction, many amphibians display concurrent elevations in plasma levels of corticosterone with no suppression of reproductive behaviour or physiology. The positive relationship between corticosterone and reproduction are evident with the onset of the reproductive season and with the energetic demands of the reproductive biology. During reproduction, it may be necessary for an animal to attempt to endure the stress by down-regulating the HPA axis. This may maximise reproductive success although it may increase mortality. Adult females in the reproductive state are more likely to suppress adrenocortical responses in comparison to breeding males and non-breeding females. All this becomes important when monitoring changes in hormonal levels during the breeding cycle and to take note of what may affect the results during the study (Moore, 2003).

During the present study, the increase in the concentration of sex steroid hormones in plasma coincided with the start of the breeding season and in particular, the development of the gonads and secondary sex characteristics. Again, much variation existed between the three study sites, but all showed the same trends. There was a decrease in testosterone and oestrogen concentrations after the first rain and a small increase leading to the second rainy spell. This would again suggest that two breeding periods exist or that breeding in X. laevis is strongly linked to environmental factors such as rainfall and that animals are opportunistic breeders,

Some ecological aspects of X. laevis breeding were also observed. Information on amphibian populations in decline is often not available (Richter, 2003; Pickford, 2003 and Tavera-Mendoza, 2002). It comes down to the following question. Why do some amphibians persist for long periods of time whereas others do not? Answering this question requires comprehensive information on both reproductive and population ecology, data that are often difficult to obtain for declining species. Amphibian ecology

studies have relied on indirect measures, e.g. egg mass counts to estimate demographic variables such as population size and reproduction success (Gilbert, 1994). Pickford (2003) stresses the need for more comprehensive ecotoxicological test methods for at-risk amphibian groups. Amphibia are important predators and prey components of aquatic and terrestrial ecosystems and need to be studied in more detail. *Xenopus* are more or less non-selective predators (Tinsley *et al*, 1996). They will feed on small crustaceans, insects, fish, amphibians and birds (Marx, 1961). The offspring of *Xenopus* may also make a significant contribution to the nutrition of the parental population (Hey, 1949 and McCoid & Fritts, 1980). Tadpoles make use of a highly specialised filter feeding mechanism to extract the finest material in suspension. Cannibalism enables the adult to exploit the nutrient resource, which could not be utilised directly. *Xenopus* also form part of the diet of many other animals. Loveridge (1953) found *Xenopus* in the stomach of a hammer-headed stork and an otter (Loveridge, 1942). Predatory fish like the largemouth bass and, in South Africa the catfish also prey on *X. laevis* (Hey, 1949).

An important factor in ecology studies may be the size of the pond (Oertli, 2002). Does size matter? Does a large area support more species? Are many small or few large ponds better? These questions must be kept in mind when looking at the conservation value of a pond. Oertli (2002) stated that the biogeography principle that larger areas support more species seems to have limitations in its application to ponds. He found that ponds of small size had more species and had a greater conservation value than a single large pond of the same total area. Nevertheless, large ponds cannot be ignored in conservation policy, since they can harbour species missing in smaller ponds. Many species live in both large and small ponds, and larger populations will live in larger ponds and will be less sensitive to extinction.

The mark-and-recapture exercise conducted during the present study was reasonably successful. The variations observed in the mark-and-recapture study were partially due to the owner cleaning out the pond. In one instance, three first-time captures drowned when water was let into the pond and the traps were subsequently submerged. An

increase in recaptured individuals and a decrease in unmarked individuals was expected, but the percentage recaptures fluctuated significantly throughout the study. The pond was situated near the large Potchefstroom dam and migration between the two sites cannot be ruled out, especially when the pond was cleaned out. Although no predation was witnessed, it cannot be ruled out especially since the pond had clear water. Large schools of tadpole were observed and juveniles were frequently trapped The positioning of the traps did not determine the capture success of the study. There was no statistical significance between northern or southern traps, nor were there between the eastern and western traps. For amphibian conservation, is it important to protect all water bodies, however small they may be. It became clear that ponds do not always sustain large numbers of *Xenopus* and populations can easily be overestimated. In areas where *Xenopus* is harvested, care should be taken not to over-exploit resources. Although *Xenopus* are very common in South Africa, over-exploitation could disrupt the ecological balance in a particular water body.

Finally, it became clear from this study that very little is known about the basic biology of wild *X. laevis*. This likely applies to most other amphibians as well. It is important to gather this knowledge before trying to investigate ecotoxicological effects on amphibians. No conclusive effect of atrazine was evident during this study. Further research is needed in regions where absolutely no atrazine is used and where endpoints, such as gonadal anomalies, can be determined. It was confirmed that *X. laevis* have an extended breeding season from August to March, but that reproduction was mainly controlled by rainfall during the preceding days, suggesting that *X. laevis* are opportunistic breeders.

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