REPRODUCTIVE CYCLE AND SEXUAL SIZE DIMORPHISM OF THE NILE CROCODILE (*Crocodylus niloticus*) IN THE OKAVANGO DELTA, BOTSWANA

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DECLARATION

I, the undersigned, hereby declare that the work contained in this thesis is my own original work

and that I have not previously in its entirety

or in part submitted it at any university for a degree

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Date: 24/11/2006

ABSTRACT

The reproductive cycle of female and male *Crocodylus niloticus* was investigated in the Okavango Delta, Botswana.

Plasma samples collected from crocodiles larger than 110 cm in total length (TL) were analysed for: (i) estradiol-17β (E_2), testosterone (T) and progesterone in females, and testosterone in males by means of enzymatic immunoassays (EIA); (ii) vitellogenin (Vtg), by one-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis; and (iii) triacylglycerol (TAG), total cholesterol (CHO) and phospholipids (PL) by means of colorimetric enzyme assays. In addition, sperm samples were collected from males larger than 180 cm TL and observed under a microscope for the presence or absence of spermatozoa.

Females reach sexual maturity at 230 cm TL. Only 60 % of adult females reproduced each year. Reproductively active females produced plasma vitellogenin during winter and early summer (June-September), when elevated plasma steroid E_2 concentrations were detected. Sex steroid hormone concentrations were comparable between reproductively and non-reproductively active females, however they did not follow the same cyclicity. Non-reproductively active females showed elevated hormone concentrations during August instead of June, and did not produce significant levels of plasma Vtg.

One male of 192 cm TL was producing spermatozoa, therefore, male *C. niloticus* may reach sexual maturity at this size. However this size class may not be able to successfully reproduce because of competition with larger males. Spermatozoa were present in the semen samples from June, when plasma T started rising, throughout October. Plasma T peaked in August during the mating season.

Plasma TAG, CHO and PL, neither varied seasonally, nor correlated with seasonal variation in any plasma steroid hormone concentrations or reproductive events either in females or males.

Variation in plasma sex steroid hormone and lipid concentrations in different size classes was also investigated.

In addition, four body and three head measurements were analysed for Sexual Size Dimorphism (SSD). The head shape of adult males and females differed significantly, males having a broader and shorter head than females. Baseline information on growth form and equations predicting Snout-Vent Length or TL from any trait measurements are also available.

UITTREKSEL

Die voortplantingssiklus in wyfies en mannetjies van *Crocodylus niloticus* in die Okavango-delta, Botswana, is ondersoek.

Plasma-monsters, versamel van krokodille met 'n totale lengte (TL) groter as 110 cm, is geanaliseer vir: (i) estradiol-17β (E_2), testosteroon (T), progesteroon (P) in wyfies en testosteroon in mannetjies m.b.v. ensiem-immuno-essaiëring (EIE); (ii) vitellogenien (Vtg) m.b.v. eendimensionele sodiumdodesielsulfaatpoli-akrielamied-gel-elektroforese en (iii) triasielgliserol (TAG), totale cholesterol (CHO) en fosfolipiede (FL) m.b.v. kolorimetriese ensiem-essais. Sperm van mannetjies, groter as 180 cm TL, is ook versamel en onder die mikroskoop bestudeer vir die teenwoordigheid of afwesigheid van spermatosoë. Wyfies bereik seksuele volwassenheid by 'n TL van 230 cm. Slegs 60 % van volwasse wyfies het voortgeplant elke jaar. Seksueel-aktiewe wyfies produseer plasma-vitellogenien gedurende die winter en vroeë somer (Junie-September), wanneer verhoogde plasma-steroïede (E_2) waargeneem is. Geslagssteroïed-hormoonkonsentrasies is vergelyk tussen seksueel-aktiewe en seksueel-onaktiewe wyfies, maar hulle het nie dieselfde siklus getoon nie. Seksueel-onaktiewe wyfies het verhoogde hormoonkonsentrasies getoon gedurende Augustus i.p.v. Junie, en het nie beduidende vlakke van plasma-vitellogenien geproduseer nie.

Een mannetjie met 'n TL van 192 cm het spermatosoë geproduseer. Dit is dus moontlik dat *C. niloticus*-mannetjies seksuele volwassenheid by hierdie grootte bereik. Hierdie grootteklas mag dalk egter nie in staat wees om suksesvol voort te plant nie, weens kompetisie met groter mannetjies. Spermatosoë was teenwoordig in die semenmonsters geneem in Junie, wanneer die plasma-testosteroon begin styg het tot regdeur Oktober. Plasma-testosteroon het 'n piek bereik in Augustus gedurende die paarseisoen. Geen seisoenale variasie is opgemerk in plasma TAG, CHO en FL, asook geen korrelasie met seisoenale variasie in enige van die plasma-steroïedhormoonkonsentrasies of voortplantingsverskynsels in wyfies of mannetjies nie. Variasie in die konsentrasie van plasma-geslagssteroïedhormone en plasma-lipiede is ook ondersoek in die verskillende grootte-klasse.

Vier liggaams- en kopmates is ook geanaliseer vir geslagsdimorfisme. Die vorm van die kop in volwasse mannetjies verskil aansienlik van dié van volwasse wyfies. Mannetjies het 'n breër en korter kop as dié van wyfies. Basiese inligting oor die groeivorm en vergelykings wat die snoet-kloaaklengte of TL bereken vanaf enige tipiese mate, word ook verskaf.

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AN OVERVIEW OF THE REPRODUCTIVE CYCLE OF REPTILES.

1.1 REPRODUCTIVE ORGANS OF REPTILES AND GAMETOGENESIS

The gonads of both sexes lie in the posterior part of the abdominal cavity, near the kidneys, and are suspended by mesenteries. In snakes and snake-like lizards they are markedly asymmetrical in position, the right being further anterior than the left. In certain burrowing snakes, the Typhlopidae, only the right ovary is well developed, the left one together with its oviduct having practically disappeared (Gabe & Saint Girons, 1965). The gonads of all vertebrates have two functions. Firstly they produce the gametes (eggs and sperm) and secondly they also function as glands for the production of hormones, which have an important role in the regulation of reproduction.

1.1.1 Male reproductive organs and spermatogenesis.

In lizards and snakes, the male copulative organ is formed by two hemipenis', the two arms of which are caudal extensions of the cloaca, each of which can be inserted independently of the other into the cloaca of the female. During copulation, the sperm runs along an external groove (Bellairs, 1969). Spines or ridges help to maintain intromission. The penis of chelonians and crocodiles is a modification of the floor of the cloaca. It consists of a median groove with ridges of erectile tissue along each side. The caudal end of the penis is a raised gland, which acts as an entering wedge prior to the filling of the erectile blood sinuses. However, the Tuatara (*Sphenadon punctatus*) is unique in possessing no male organ of intromission, where the sperm is transferred by direct cloacal contact as in many birds.

The color and shape of the testis of reptiles varies significantly. They are particularly elongated in snakes and snake-like lizards. In *Vipera berus* they are yellowish-white and cylindrical, and situated in the caudal third of the body (Volsoe, 1944). The oval white testis of *Xantusia vigilus* becomes greyish in summer (Miller, 1948). Testes of chameleons are black (Bourgat, 1970). They can be multi-lobed as in the snake *Leptotyphlops humilis* (Werner & Drook, 1967). The testes of immature *Crocodylus palustris* are small, white, rod-like bodies (Ramaswami & Jacobi, 1965).

Generally speaking the testes of reptiles are similar to those of birds and mammals and are vascularized by spermatic arteries and veins (O'Donoghue, 1912; Harris, 1963; Duda, 1972).

Spermatogenesis takes place in the tubular compartments, the seminiferous tubules, which are invested by a basal lamina and a more or less well-developed connective tissue and contain primarily cells of the germinal line at different stages of spermatogenesis. The sertoli cells are the only somatic cells within the seminiferous tubules.

Only two early histological studies in reptiles comprehensively describe the morphology of germ cells as they progress through the phases of spermatogenesis, namely, in the viper (Volsoe, 1944) and the garter snake (Fox, 1952). The regenerative phase usually involves spermatogonial division with little testicular enlargement, which occurs at the very beginning of recrudescence of spermatogenesis. The proliferative or progressive phase normally involves further spermatogonial divisions and meiotic activity up to the early spermatid stage (round spermatids), which typically results in appreciable enlargement of the testis. The last phase of spermatogenesis, the culminative phase, is characterized by the development of advanced spermatids and mature spermatozoa.

1.1.2 Female reproductive organs and oogenesis.

The ovaries of reptiles are either oval (in most lizards and turtles) or elongated (as in snakes) structures attached to the dorsal body wall by a mesovarium. The ovarian cortex, consisting of sparse stroma and follicles, is covered by a squamous epithelium (Jones, 1978). The ovaries of most reptiles are paired, however, one ovary fails to develop in a few species (Franchi, 1962). Some species also have structures for storing sperm in the oviduct. In snakes, turtles and lizards, sperm are retained in seminal vesicles, which are specialized alveolar glands at the base of the infundibulum. Ducts lead into the oviductal lumen from these glands (Fox, 1977).

Oogenesis, the formation of oocytes from oogonia, occurs throughout the reproductive life of reptiles, in contrast to the Petromyzontia, Elasmobranchii, a few Teleostei, Aves and Mammalia, in which oogenesis is restricted to the embryonic phase. The oogonia divide mitotically, eventually forming oocytes which are then surrounded by follicle cells, forming primordial follicles (Guraya, 1989). The accumulation of yolk material in the oocyte, the last phase of vitellogenesis, is one of the most conspicuous features of the reptilian ovarian cycle, and is usually used to define seasonal patterns in ovarian activity. During vitellogenesis yolk is synthesized and secreted by the liver under the influence of estrogen produced by the wall of maturing follicles, and is then transported to the ovaries and incorporated into the oocyte. Once the oocyte has attained its full growth during the vitellogenic phase of oogenesis, it is ready for the next phase, the resumption of reduction division for the final maturation which

is the germinal vesicle breakdown (GVDB), chromosome condensation and extrusion of the first polar body. GVDB leads to the intermixing of the nuclear content with the surrounding cytoplasm. The oocytes are arrested at the prophase of the first meiosis when synthetic activities are very high. This process is called maturation (Guraya, 1989). Ovulation is the process by which the mature ovum is released by the rupture of the follicle wall at its apex. Preovulatory gonadotrophins, especially a luteinizing hormone (LH) surge, bring about ovulation. It is known that the oviductal period of eggs and the luteal phase precede oviposition. Follicle atresia may occur at all stages of follicular development (Kuchling, 1999).

1.2 BREEDING CYCLES

Many vertebrates reproduce periodically, meaning that there is an alternation between periods of reproductive activity and inactivity. These phases frequently follow changes in environmental factors and this is particularly evident in higher and mid-latitudes where the reproductive phases are coupled with the changing of seasons. The reproductive phases are in turn coupled with cyclical changes in the gonads. Without exception, reptiles inhabiting temperate latitudes show some degree of seasonality in reproductive physiology in both sexes. There is invariably some seasonal hiatus in gametogenesis and probably steroid secretion (Licht, 1984). Even at lower latitudes, including low altitude tropical regions, many species have a pronounced seasonality in gonadal activity (Saint Girons & Pfeffer 1971; Angelini & Picariello 1975, Voris & Jayne 1979). However, there are also numerous reports or reviews which conclude that certain reptilian species, especially in the more equable and humid tropics, are "continuous" breeders (Saint Girons & Pfeffer, 1971; Barbault, 1974, Angelini & Picariello, 1975).

Reptiles display three fundamental reproductive patterns, based on the degree of variation in gonadal activity throughout the year: (i) continuous or acyclic reproduction (aseasonal) with roughly similar levels of gonadal activity in all months, (ii) continuous or cyclic reproduction (aseasonal) but with gonadal activity fluctuating across months and (iii) discontinuous or seasonal reproduction where periods of gonadal activity alternates with periods of gonadal quiescence (Licht, 1984; Callard & Kleis, 1987).

In some species, spermatozoa can have an unusually long functional survival time in the reptilian female reproductive system; therefore, synchronization of ovulation and copulation are not essential

1.2.1 Males

There are at least two different aspects of the male urinogenital system that must be considered to evaluate the state of reproductive activity, namely, spermatogenic condition and endocrine activity. These two aspects are not necessarily synchronous, which can be a problem when assessing the degree of activity from a "steady-state". For example: histological observation may reveal the presence of sperm, but does not provide an accurate index of rate of sperm production (Litch, 1984).

Continuous breeders show spermatogenic activity throughout the year (Saint Girons & Pfeffer, 1971; Barbault, 1974; Angelini & Picariello, 1975) but several types of evidence suggest some degree of seasonal variation in testicular activity in many of the presumptive continuous breeding species. Despite year-round spermatogenic activity, significant seasonal cycles in testis mass occur (Licht & Gorman 1970, Ruibal *et al* 1972, Gorman & Licht 1975, Sexton *et al*., 1971). These changes in testes mass suggest seasonality in the rate of sperm production. A second relevant aspect is androgen production. Even though spermatogenesis may appear to be continuous, pronounced seasonal cycles in the levels of plasma androgen or the histological appearance of androgen secreting Leydig cells have been reported (Gorman *et al*., 1981; Del Conte, 1972).

Without exception, reptiles inhabiting temperate latitudes show some degree of seasonality in reproductive physiology (Licht, 1984).There are two common types of seasonal cycles seen in temperate species of reptiles: (i) the postnuptial cycle and ii) the prenuptial cycle.

In the postnuptial cycle, exhibited by most turtles and some snakes, recrudescence of spermatogenesis does not occur until after spring mating has taken place. Testicular enlargement and spermatogenic activity take place before winter. Sperm is then stored in the epididymis and the testis enters a quiescence period that lasts the entire winter (Licht, 1984).

In the prenuptial cycle, reptiles produce sperm just before the mating season. Most snakes and lizards exhibit this type of germ cell cycle (Saint Girons & Kramer, 1963; Mayhew, 1968; Licht and Gorman, 1970; Saint Girons, 1972; Angelini & Picariello, 1975; Saint Girons, 1976).

1.2.2 Females

The greater energy investment involved with egg production and, in some cases, the time investment in post-ovulatory development (viviparity or brooding) make it unreasonable to expect constant gamete production in females. The closest approximation to this condition would be those species, for example, the anoline lizards and geckos, in which many single

clutches can be produced at relatively short intervals. At the opposite extreme are some species, such as the large sea turtles, which may produce one or several very large clutches in one breeding period but then breed and nest only every second, third or fourth year (Hirth, 1971; Moll, 1979). Thus, seasonality in female reptiles is better defined at the level of the population than the individual (Litch, 1984).

In species with a continuous pattern, ovulation occurs in all seasons, and species exhibiting this pattern have short ovarian cycles throughout the year. For example: many tropical lizards and a few tropical snake species (Fitch, 1970; Sherbrooke, 1975). In other tropical and subtropical species, the pattern of ovarian activity can be continuous with variation in reproductive activity. With this type of ovarian activity, there is some variation in the percentage of ovigerous females during the year, but the height of breeding activity is not totally predictable and seems to be cued in many cases by rainfall (Sherbrooke, 1975). Some tropical and most temperate snakes and lizards, however, exhibit a non continuous ovarian cycle, ie: they breed at a predictable time each year. Many temperate lizards exhibit this pattern, most ovulating in the spring (Fitch, 1970). However, some temperate viviparous lizards ovulate in the fall (Golberg, 1971). Some temperate lizard species with seasonal breeding have two to several clutches within the breeding season, whereas others have but one (Fitch, 1970).

Another kind of classification of ovarian patterns in reptiles relates to evolutionary strategies (Tinkle *et al.*, 1970). Many tropical and some temperate lizards, for example, are early maturing and have multiple broods. Most of these are oviparous and have a relatively small clutch size. In contrast, many temperate lizards are late maturing and single-brooded, some are viviparous and clutch or litter size is relatively large.

1.3 REPRODUCTIVE STRATEGIES

Animals have evolved a wide range of reproductive strategies to improve their fitness. These can take place at different levels:

1.3.1 From oviparity to viviparity

Reproduction in the class Reptilia exhibits a wide variety of traits: squamates are mostly oviparous, but have evolved ovoviviparity and viviparity over time (Shine, 1985);

parthenogenesis also occurs in squamates (Crews & Moore, 1989) and highly developed parental care occurs in crocodiles and in some other squamates (Shine 1988).

As far as the development of the embryo is concerned, a distinction can be made between, oviparous, ovoviviparous and viviparous forms. In oviparous forms, the young emerge outside the mother's body from eggs; in ovoviviparous species the mother gives birth to live young which emerge from eggs containing enough yolk to nourish the embryo during its development but in which no nutritive connections between mother and young exist. In viviparous animals young are born after a term of intimate nutritive connections between embryo and mother (Amoroso, 1952). These patterns can be viewed as "reproductive strategies", that have advantages as well as disadvantages that affect evolution.

Oviparity is generally thought to be the ancestral mode of reproduction in reptiles, with viviparity being the derived condition (Neill, 1964; Shine, 1985). Viviparity in reptiles is thought to arise through a gradual increase in the duration of uterine egg retention, culminating in the birth of fully formed offspring (Packard *et al.*, 1977; Shine & Bull, 1979; Guillette, 1993). Egg retention could be beneficial in unpredictable or extreme climates because it protects the embryos from exposure to potentially harmful nest conditions, such as extremes of temperature or moisture (Qualls & Shine, 1998).

1.3.2 Clutch and egg size

In most reptiles, egg size and clutch size are positively correlated to maternal body size (Rowe, 1994; Shanbhag *et al.*, 2000; Radder & Shanbhag, 2003; Shine, 2003) and there is often a trade-off between egg size and clutch size. The larger the clutch, the smaller the eggs (Rowe, 1994), however, some species do not exhibit any trade-off between these two life-history traits (Radder & Shanbhag; 2003).

Some reptiles, such as *Calotes versicolor* (Agamidae), have evolved several strategies to optimize its reproductive fitness such as plasticity to manipulate clutch and egg size depending on the time of breeding (Shanbhag, 2003). Larger eggs, produced late in the breeding season, produce heavier hatchlings and also contain more internalized yolk than those that hatch earlier in the breeding season, thereby enhancing fitness of the hatchlings which will have to compete for resources with older individuals of early clutches (Radder *et al.*, 2002).

1.3.3 Sperm storage

Several species of reptiles are known to store sperm in the oviduct for variable lengths of time depending upon the species (Shanbhag, 2002). This phenomenon is essentially found among temperate species in which the gonadal cycles are temporally dissociated (males producing sperm in autumn and mating with females prior to ovarian recrudescence) and often in species which hibernate (females, after emerging from hibernation, ovulate and use the stored sperm for fertilization).

Some reptiles, such as tropical lizards, which do not present a dissociated gonadal cycle and do not hibernate, can also store sperm (Shanbhag, 2003). However, these species usually produce multiple clutches, which suggests that an oviductal sperm storage strategy has evolved to eliminate repeated mating and reduce risk of predation.

1.3.4 Age/size at sexual maturity and frequency of reproduction

Due to the difficulties associated with the aging of animals in the wild, studies often use size instead of actual age to describe growth patterns and age/size at sexual maturity. Most vertebrates begin reproducing before they attain their maximum body size. Prior to maturation, energy is allocated to maintenance and growth, whereas after maturation it is also allocated to reproduction. In chelonians, the general growth pattern is a rapid juvenile growth phase until maturity followed by a continuous, slow adult growth phase (Andrews, 1982; Wilbur & Morin 1988). A slight decrease in growth rate can be observed prior to maturation which might reflect a physiological preparation for first reproduction, especially in females. In addition, males and females may reach sexual maturity at different ages/sizes.

Frequency of reproduction usually differs between male and females due to the sexual difference in energy costs, which are much more elevated in females than males. For instance, wild female crocodilians reproduce once every 2 to 3 years (Graham, 1968; Lance, 1989; Kofron, 1990; Guillette *et al.*, 1997), whereas males may reproduce every year.

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CHAPTER 2

REPRODUCTIVE PHYSIOLOGY OF FEMALE NILE CROCODILE (*CROCODYLUS NILOTICUS*) IN THE OKAVANGO DELTA, BOTSWANA.

2.1 INTRODUCTION

Understanding reproductive patterns and their hormonal control in animals is indispensable for structuring proper management strategies for aquatic and terrestrial ecosystems, especially regarding key stone species such as crocodilians (Magnuson *et al.*, 1990).

Previous studies of reproductive biology of the order Crocodilia, in wild populations, primarily involved investigations of reproductive ecology, such as clutch size, hatchling success, reproductive frequency and reproductive cyclicity (Cott, 1961; Graham, 1976; Pooley, 1982; Lance, 1989; Thorbjarnarson, 1994; Leslie 1997). Studies investigating physiological aspects of reproduction have focused primarily on the American alligator, *Alligator mississippiensis*, in which seasonal variation in plasma sex steroid concentrations of different populations were well studied (Lance, 1989; Guillette *et al.*, 1997; Rooney *et al.*, 2004), as well as the effect of environmental contaminants on hormone levels (Guillette *et al*, 1994; Guillette *et al*, 1996; Crain and Guillette, 1997; Guillette *et al.*, 2000).

With regards to the Nile crocodile, *Crocodylus niloticus*, only one comprehensive study describing the reproductive cycle of a wild population in Zimbabwe has been published (Kofron, 1990). This study determined that females reached sexual maturity at approximately 262 cm in total length (TL). Follicle growth and vitellogenesis commenced with a decrease in air temperature and an increase in plasma estrogen concentrations in April (end of summer). Estrogen and testosterone concentrations peaked in June-July (mid-winter). Ovulation occurred in August, after mating, which was followed by a decrease in estrogen concentrations and oviposition three weeks later in September (spring). Females attended to nests during the entire incubation period, which lasted until mid-December when eggs hatched. An important feature in the female reproductive cycle which was not investigated in Kofron's study (1990) is vitellogenesis.

Vitellogenesis is an important event in the reproductive cycle of all oviparous vertebrates. It is a multi-step process where vitellogenin (Vtg), a major egg yolk protein precursor, is synthesized and secreted by the liver and transported in the blood to oocytes, where it is

absorbed and cleaved to formed egg yolk polypeptides such as lipovitellins and phosvitin (Bergink *et al*, 1974; Wallace, 1985; Ho, 1987). Vitellogenin is normally found in the plasma of sexually mature females undergoing vitellogenesis. However, the liver of both reproductively immature females and males is able to synthesize Vtg in response to estrogen stimulation (Jackson *et al*., 1977; Ho *et al*., 1981; Palmer and Palmer, 1995). A few studies have demonstrated seasonal changes in plasma lipid components which can be related to changes in plasma steroid hormone concentrations throughout the reproductive cycle (McPherson and Marion. 1982; Hamann *et al*., 2002; Duggan *et al*., 2001; Lance *et al*., 2002) The lipid composition of yolk of freshly laid eggs of reptiles has been reported for alligators and a number of turtle species (Noble *et al*., 1990; Rowe *et al*., 1995; Lance *et al*., 2002). The yolk contains 68 - 79 % of triacylglycerol (TAG), 7.2 - 21.4 % of phospholipids (PL) and 4.3 - 9.16 % of total cholesterol (CHO). The origin of the various lipid fractions in yolk is at present unclear, but most are the result of uptake of lipoproteins from the blood by the developing oocyte.

The objective of this study was to investigate changes in circulating hormone levels and vitellogenin during the natural reproductive cycle of female *C. niloticus* in the Okavango Delta, Botswana and to describe the ontogenetic shift in plasma sexual hormone concentrations. In addition, this study investigated the variation in plasma lipid components throughout the year and related these changes to seasonal changes in steroid hormone concentrations. A management plan is urgently required for the Okavango crocodile population and this study will provide essential baseline data regarding the reproductive endocrinology of wild Nile crocodiles in the Okavango Delta. This will in turn assist crocodile farmers, throughout Africa, with regards to improving productivity and therefore the sustainable utilization of crocodiles.

2.2 MATERIAL AND METHODS

2.2.1 Study area and Climate

The Okavango River is shared by three countries and has a catchment area of more than 300 000 km^2. The river rises in the Angolan Highlands as two tributaries, the Cubango and the Cuito rivers, and after several hundred kilometres, enters Botswana from the north where it forms a very broad floodplain known as the Panhandle, which eventually fans out to form the Okavango Delta (Figure 1). The Panhandle and its riverine floodplain, together with the upper part of the Delta, forms a permanent swamp while the lower parts of the Delta are seasonal floodplains. The size and timing of the annual flood depends more on rainfall in the catchment area (Mendelsohn and El Obeid, 2004) as opposed to local rainfall. As soon as the Okavango River leaves the Panhandle area, it spreads out over the sands of the Kalahari forming a wide, fan-shaped delta. The northern part of the Delta is characterized by shallow water, flooded grasslands, backwater swamps, ox-bow lakes and many hidden lagoons, mostly interconnected by narrow waterways. Only a few main channels lined by tall reeds (mainly *Phragmites australis*), carry the remainder of the Okavango's water southwards through the Delta.

The climate of the area is sub-tropical with hot, rainy summers and warm, sunny and dry winters. Temperatures vary from an average maximum of 30-35° C from October to January to 7°C in the coldest months of June and July. The rainy season starts in November and ends in February (Figure 2 from Mendelsohn and El Obeid, 2004). Another feature of the Okavango Delta is the flooding regime of the river. In the Panhandle region the floods start at the end of February and the water starts receding in June. The peak flows enter Botswana in April. During the height of the flooding season, crocodiles disperse into the floodplains, probably to follow the fish and find dry ground for basking during the colder winter months, which makes it difficult and often impossible to capture them using current capture techniques (pers. comm. AJ Leslie).

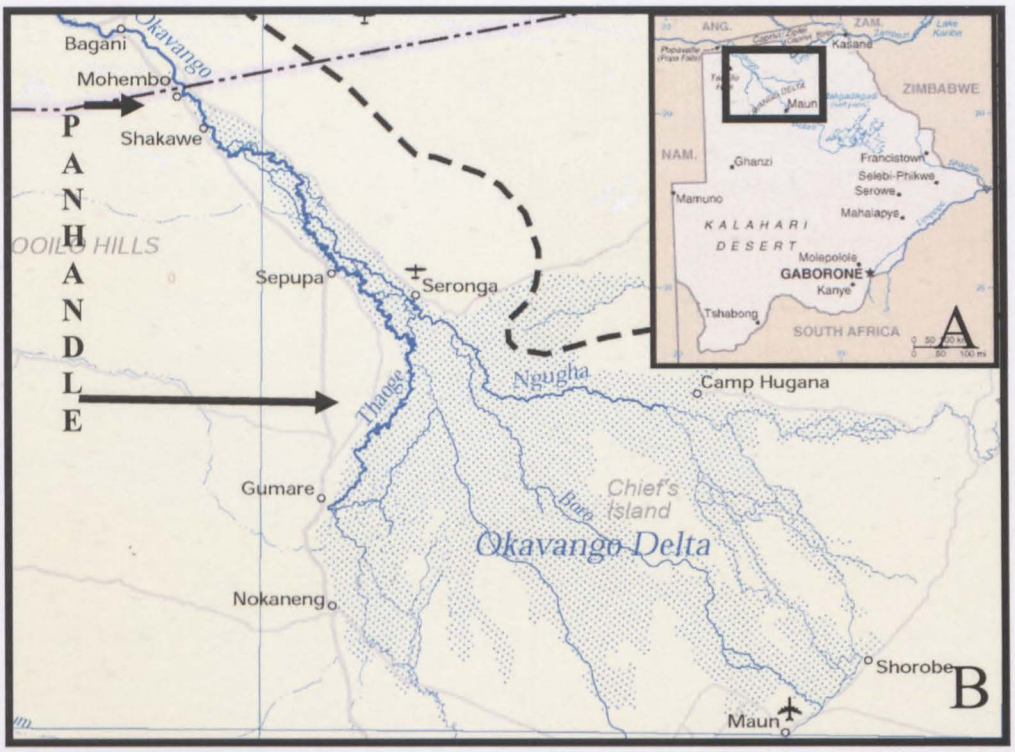

Figure 1. The Okavango Delta is situated in the northwest of Botswana (A). Nile crocodiles were captured in the Panhandle area (B). Adapted from CIA World Factbook.

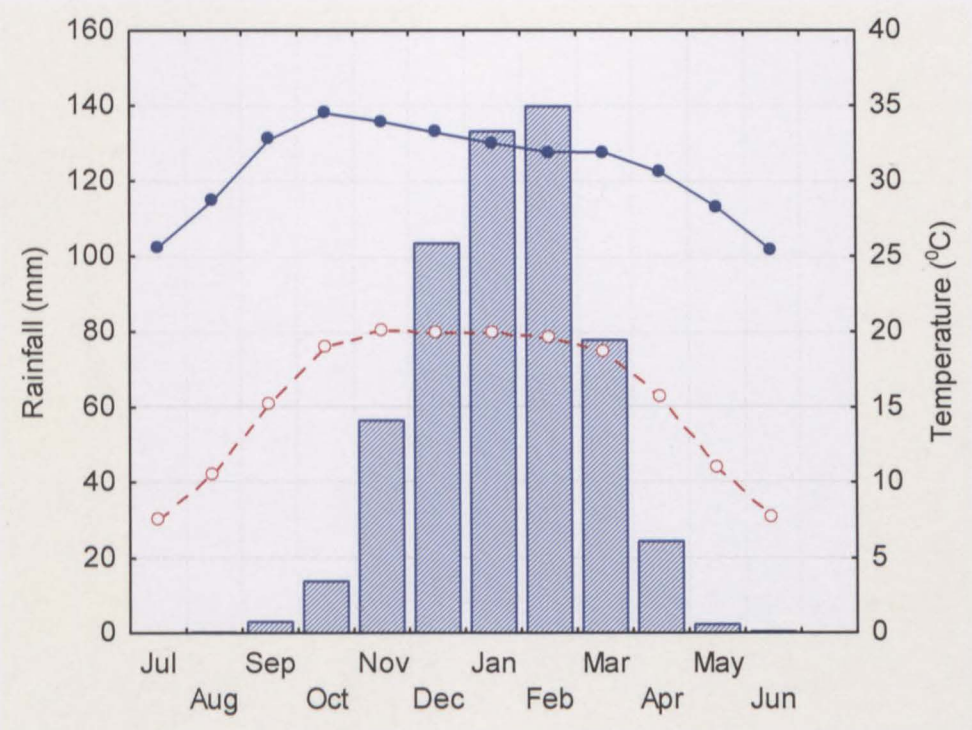

Figure 2. Total monthly rainfall (bar graph), maximum ambient air temperatures (closed circles) and minimum ambient air temperatures (open circles), recorded at Shakawe, Botswana.

2.2.2 Study organism

Crocodylus niloticus (Laurenti, 1768) belongs to the class Reptilia, order Crocodilia, suborder Eusuchia, family Crocodylidae, and subfamily Crocodylinae. Crocodilians belong to the great group of archosaurs ("ruling reptiles") which also included the extinct thecodonts, its earliest and most primitive members, the dinosaurs, the pterosaurs or flying reptiles, and the ancestors of birds (Benton, 1982). Crocodilians of the suborder Eusuchia appeared in the Cretaceous period (140 to 65 million years ago) and all living crocodilians belong to this suborder. They occupy an important position in the evolution of vertebrates as they exhibit anatomical features found only in dinosaurs and features common to birds and mammals (Brochu, 2001). There are currently 23 species of crocodilians all located between the latitudes of cancer (23.5° north) and Capricorn (23.5° south). The Nile crocodile is one of three species living on the African continent, the two other species being the dwarf crocodile (*Osteolaemus tetraspis*) and the long-snouted crocodile (*C. cataphractus*).

The Nile crocodile is unevenly distributed throughout the Okavango system, although the majority of the breeding population is found in the Panhandle, where permanent water is available.

2.2.3 Capture methods

Two capture methods were used: (i) At night, using a 4.8 meter flat bottomed aluminium boat propelled by a 60 hp engine, crocodiles were located using a 500 000 candle light power spot light which, once shone into the crocodile's eyes, reflected back a red glow due to the presence of a retinal tapetum. Once spotted, the beam of light remained on the crocodile's eyes so as to mesmerize it, making it possible to approach the animal by boat. Crocodiles estimated to be smaller than 120 cm were captured by hand. Crocodiles between 120 and 230 cm were captured using a swivelling noose (Animal Handling, Co., South Africa) which was placed over the snout and pulled tight in the neck region. Crocodiles were then brought onto the boat, jaws were taped shut and the animals were physically constrained. Animals larger than 230 cm, were captured using a noose attached to a climbing rope, which was secured to the boat. The crocodile was allowed to swim so as to tire it out before it was brought onto the boat. (ii) During summer months, baited box and Pitman traps were strategically placed on river banks. Traps were baited at sunset and checked at first light the next morning. Captured animals were immediately restrained and the necessary data collected. No chemical mobilization took place.

2.2.4 Sampling techniques

Crocodiles were captured in the Panhandle area (Figure 1) every month between January 2002 and August 2005, except for the month of May when flooding was at a peak and crocodiles migrated into the flood plains making capture difficult. A 0.5 - 5.0 ml blood sample was drawn between 3 and 10 minutes after capture from the caudal vein in the tail (Gorzula *et al.*, 1976; Leslie, 1997). Once all data were collected the crocodile was released at the site of capture. Blood was transferred to microcentrifuge tubes (Ependorff, Germany), placed on ice, and later, when back in the field laboratory, centrifuged for 5 minutes at approximately 1500 x *g*. The plasma supernatant was aspirated, frozen at -15° C and stored at -80° C until further analysis in South Africa. Once back in the laboratory at Stellenbosch University all samples were again centrifuged with a UniEquip (Martinsred, Germany) bench top centrifuge at 4° C for 2 min at 5000 x *g* before analysis.

2.2.5 Size at sexual maturity

Total length (TL) and snout-to-vent length (SVL; ±1 mm) were recorded using a flexible measuring tape. In order to determine the size at which female Nile crocodiles reach sexual maturity, plasma samples of females with a TL greater than 180 cm were analyzed qualitatively for the presence of plasma vitellogenin (Vtg). Females with detectable Vtg were considered sexually mature. Therefore the size of the smallest female producing Vtg was considered the size at which the Okavango's female Nile crocodiles attain sexual maturity.

2.2.6 Vitellogenin analysis

The presence of Vtg was determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). A number of 1.5 mm thick Laemmli polyacrylamide vertical slab homogeneous resolving gels, containing 10 % acrylamide and stacking gel of 4.5 %, were prepared. Samples were prepared by mixing 2 µl of plasma with 10 µl PBS, 2 µl of 1 % (m/v) bromophenol blue and 25 µl of 0.125 M Tris, pH 6.8 containing 4 % (m/v) SDS, 20 % (v/v) glycerol and 10 % (v/v) mercaptoethanol. Samples (5 µl) were denatured in boiling water for 2 minutes and applied to the gell wells. Electrophoresis was performed by subjecting the gels to a constant current of 18 mA. Vtg is known to be a heavy protein of about 200 kDa (Herbst *et al.*, 2003; Selcer and Palmer, 1995; Brown *et al.*, 1997; Selcer *et al*, 2006). Electrophoresis was continued until the dye-front reached the end of the gel (≈ 2h30 minutes at room temperature). Gels were stained with 0.125 % (m/v) Coomassie Blue in 50 % methanol and 10 % (v/v) acetic acid for 2 h at 37^{0}C, and then destained with distain I (50 %

v/v MeOH, 10 % HOAc) for 2h at 37^0 C, followed by distain II (5 % v/v methanol, 7 % acetic acid) overnight at room temperature. Gels were finally digitally photographed and stored in distilled H_2O. A protein molecular weight marker was included on each gel (AEC-Amersham, South Africa). In addition, 4 plasma samples from males of different sizes (110 - 180 cm) were also analyzed in order to verify the possibility of any environmental estrogen contaminants, which may induce vitellogenin production by the liver in either males or females.

2.2.7 Plasma sex steroid hormone analysis

Plasma estradiol-17ß (E_2), progesterone (P) and testosterone (T) concentrations were measured using commercial enzyme immunoassay (EIA) test kits (International Immuno-Diagnostics, California, USA). The antibodies used were highly specific with very little cross-reactivity to other steroids (less than 2 %). Validation of the assays for crocodile plasma revealed an intra-assay coefficient of variance of less than 6 % and an inter-assay variation of less than 9 %. Plasma samples diluted with buffer solution showed good parallelism when the standard curves were prepared using the steroid samples supplied.

Tests were performed in duplicate according to the manufacturer's protocol. In brief, 25 µl of standard or plasma (diluted with E_2-HRP conjugate) were dispensed into each Goat Antirabbit IgG-coated microtiter well. One hundred µl E_2-HRP conjugate and 50 µl of Rabbit anti-E_2 reagent were added to each well and mixed. Plates were incubated at 36 ^{0}C for 60 minutes. The plates were rinsed five times with distilled water. One hundred µl of substrate (tetra-methyl benzidine) was then added to each well and incubated at room temperature for 15 minutes. The reaction was stopped with 50 µl 1 N HCl. Absorbance was measured at 450 nm, using a microtiter plate reader (Power Wave x, Bio-Tek instruments, INC).

The same protocol was used to determine P concentration except that the standards provided by the manufacturer were diluted with Progesterone-HRP Conjugate reagent. The testosterone EIA kit followed the same protocol as for the E_2 kit, except that only 10 µl of standard and plasma samples were dispensed into each well and that the first incubation at 36^0 C lasted for 75 minutes.

2.2.8 Lipid analysis

Triglyceride concentrations were determined using a colorimetric enzyme assay (Triglycerides MR, Linear Chemicals, Spain). The assay was performed following the manufacturer's protocol using ab-well microtiter plates. Samples and standards were tested in

duplicate. Absorbance was measured at 500 nm using a microtiter plate reader (Power Wave x, Bio-Tek Instruments, INC). Total Cholesterol (CHO) and Phospholipid (PL) concentrations were also determined using a colorimetric enzyme assay (Cholesterol MR, Linear Chemicals, Spain and Phospholipids, CHO-POD; Spinreact, Spain) in the same manner as for the Triglycerides and absorbances were measured at 550 nm and 505 nm, respectively.

2.2.9 Statistical analysis

Differences in plasma steroid and lipid concentrations between months, reproductive status or size class were tested using one-way ANOVA or Student t-test (Statistica 7, StatSoft, Inc. 1984/2004). Data sets were tested for normality prior to statistical analyses and were log transformed prior to testing if heterogeneity was detected. Correlations between plasma lipids and steroid hormone concentrations were determined using a Pearson's correlation analysis.

2.3 RESULTS

2.3.1 Plasma vitellogenin and Size at sexual maturity.

Vitellogenin was not detected in any male plasma samples (Figure 3). Vtg was detected in the plasma of females from the beginning of July to early October (Figure 4). The smallest female with Vtg detected quantitatively by electrophoresis, had a TL of 232 cm. Therefore, females longer than 232 cm in total length were considered to be sexually mature and referred to as "adults". Intense banding, indicative of elevated levels of plasma Vtg, was noted in samples from mid-July to early September, and during that period approximately 60 % of the adult females were producing Vtg. These females were categorized as vitellogenic whereas the others were referred to as non-reproductively active (in a particular year).

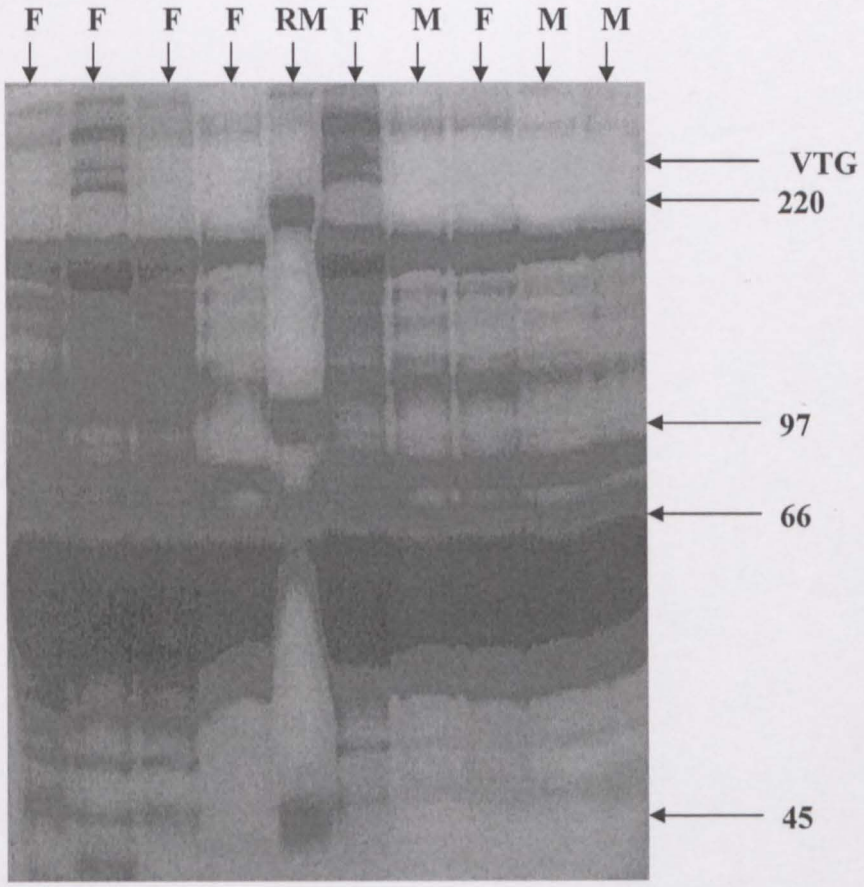

Figure 3. Denaturing gel electrophoresis of blood plasma from *C. niloticus*. Plasma proteins were separated using a 4.5 - 10 % SDS-PAGE gel and stained with Coomassie Blue R250. F = female, M = male and RM = rainbow molecular weight markers.

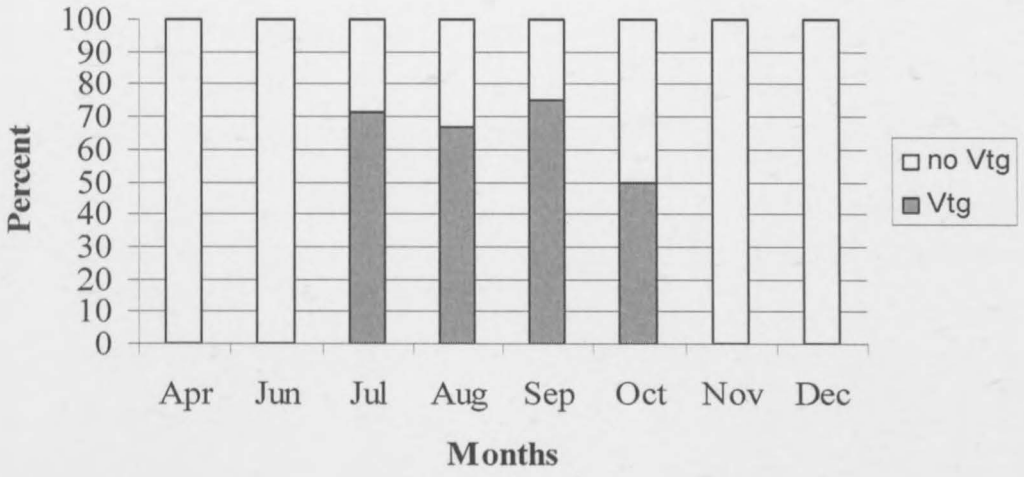

Figure 4. Monthly percentage of adult female Nile crocodiles exhibiting plasma Vtg.

2.3.2 Plasma sex steroid hormones in adult females.

Plasma estradiol. There was no significant difference in plasma E_2 between adult females (TL > 232 cm) throughout the year (Figure.5a). Although, when comparing E_2 concentrations between non-reproductively active adult females and vitellogenic adult females (Figure 6a and 6b), a significant difference was noted during the months of June-July (mid-winter) when vitellogenesis starts ($t = 3.97$, $df = 6$, $p = 0.007$). Vitellogenic females had a much higher E_2 concentration (5.16 ± 1.0 ng/ml, $n = 5$) than non-reproductively active adult females (1.93 ± 1.3 ng/ml, $n = 3$). In vitellogenic females, plasma E_2 concentrations reached a maximum in July and then declined significantly, reaching a minimum in October (1.2 ng/ml; $F = 5.86$, $p = 0.02$; Figure 5b). Plasma E_2 concentrations in non-reproductively active females peaked in August (end of winter) and then declined (Figure 6a).

Plasma testosterone. There was no significant difference in plasma T in all adult females (TL > 232 cm) throughout the year (Figure 5b). In vitellogenic females, plasma T concentrations were at their highest in July (13.82 ± 8.9 ng/ml) and declined significantly in August ($F = 3.42$, $p = 0.08$) where they maintained a basal level up until October (3.4 ± 2.32 ng/ml; Figure 6d). Plasma T concentrations in non-reproductively active females were at a basal level in June-July (2.0 ± 1.9 ng/ml) and increased significantly in August-September (19.6 ± 6.22 ng/ml; $t = 4.9$, $df = 3$, $p = 0.016$; Figure 6c) and then declined.

Plasma progesterone. There was no significant difference in plasma P in all adult females (TL > 232 cm) throughout the year ($F = 2.93$, $p = 0.08$; Figure 4c). There was no significant variation of plasma P concentrations in vitellogenic females from July (33 ± 16.9 ng/ml) to October (10.6 ng/ml) but a steady decline is noticeable (Figure 6f). In non-reproductively active females P concentrations were low in June-July (14.4 ± 15.95 ng/ml) and then rose significantly in August ($F = 1.53$, $p = 0.08$) and stayed elevated until December (43.86 ± 11.87 ng/ml; Figure 6e).

2.3.3 Variation in plasma sex steroid hormone concentrations throughout growth

Sexual hormone concentration was investigated in females of different size classes, disregarding their reproductive status. The analysis was performed on data collected from July to September corresponding to the height of the reproductive season, when sex steroid hormone concentrations were high in sexually mature females.

There was a significant variation in plasma E_2 ($F = 5.05$, $p = 0.004$; Figure 7a) between females of different size classes. As the animal grew E_2 concentrations increased from 1.28 ± 0.87 ng/ml (110-129 cm TL) to 4.88 ± 1.57 ng/ml (170-199 cm TL) and stayed at this level even when reaching sexual maturity (230 cm). A slight decline was observed in animals with TL > 270 cm but this was not significant.

There was no significant difference in plasma T concentrations between females of different size classes, but a decline was noticed when females reached sexual maturity (Figure 7b). Young (TL 110 - 129 cm) and old females (TL > 270 cm) had significantly lower P concentrations (17.13 ± 8.0 ng/ml and 20.6 ± 16.9 ng/ml respectively) than other size classes (42.14 ± 11.85 ng/ml; $F = 4.52$, $p = 0.002$; Figure 7c).

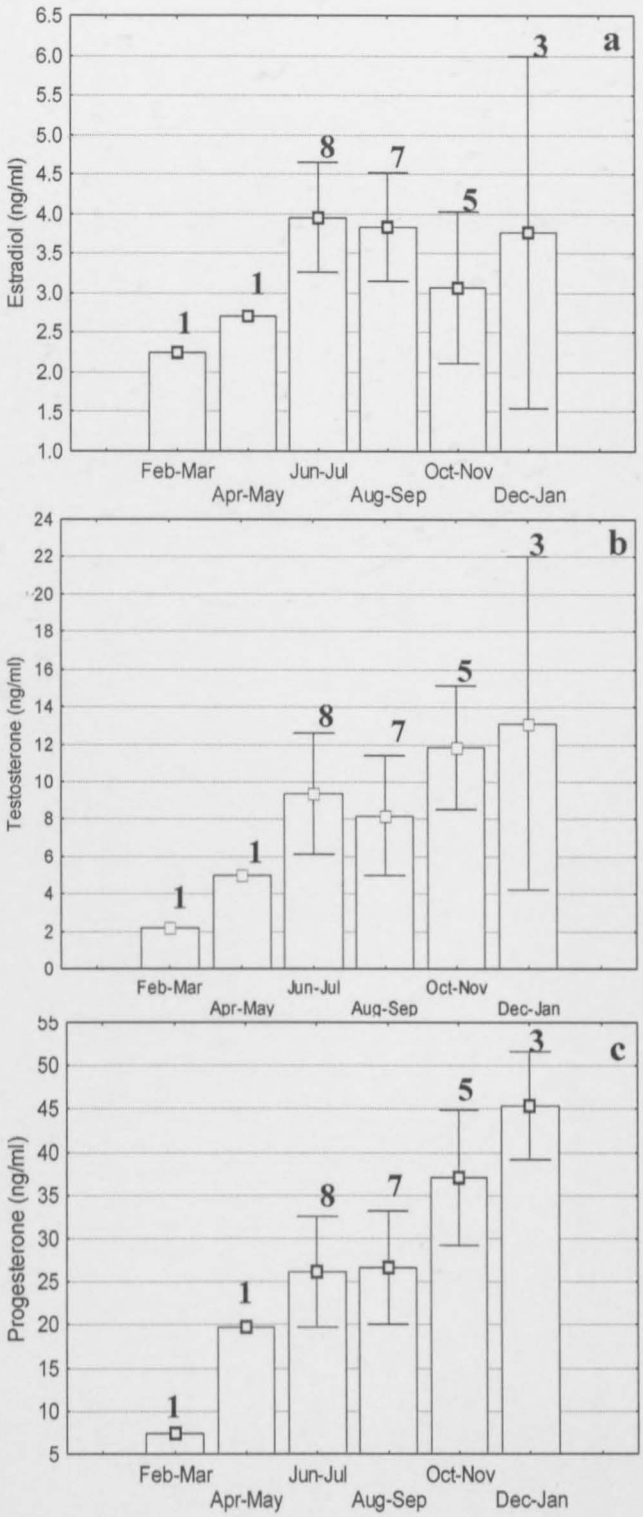

Figure 5. Monthly mean ± 1SE Estradiol-17ß, Testosterone and Progesterone concentrations in adult female *C. niloticus*. Sample sizes are given above each column.

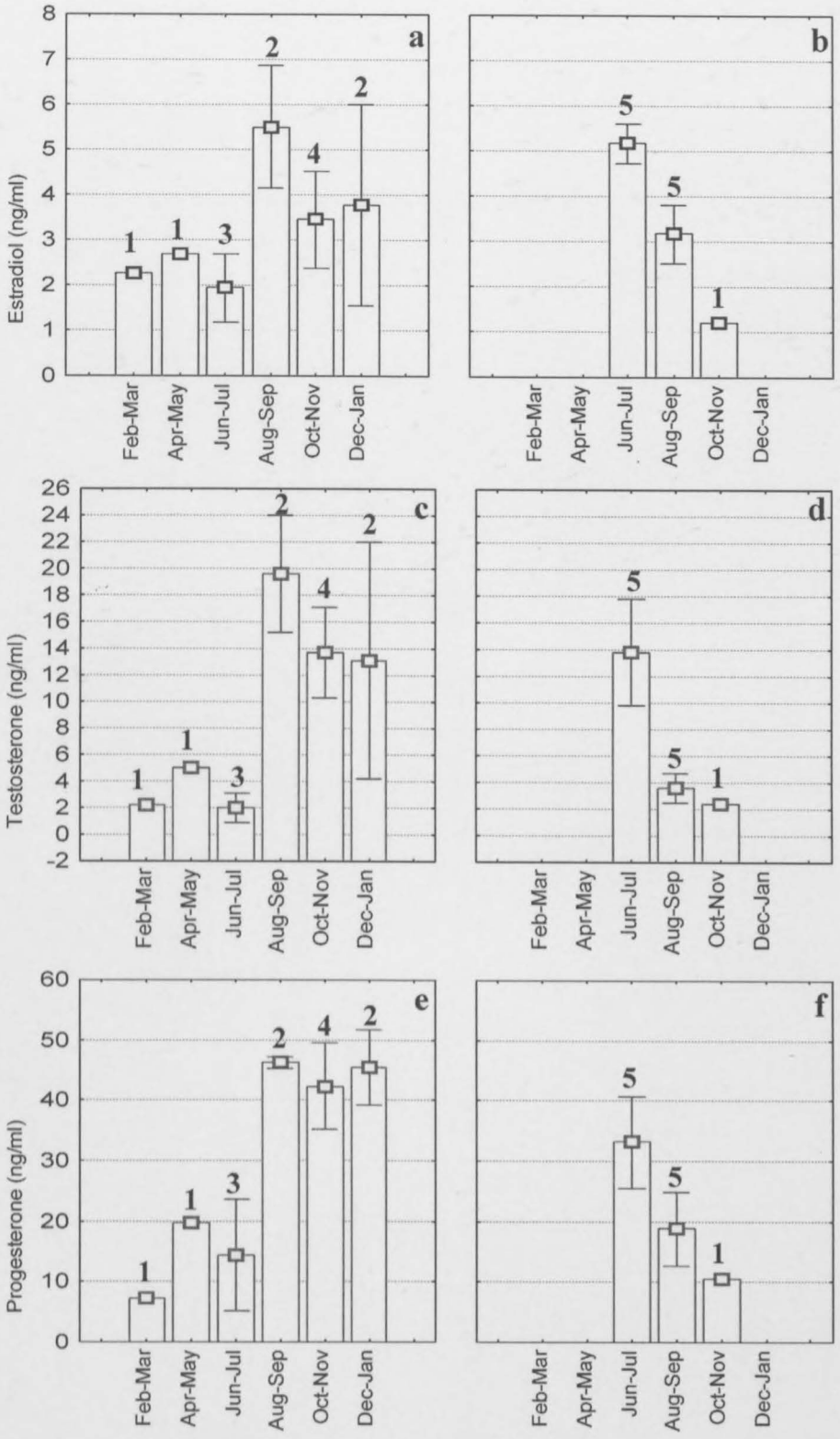

Figure 6. Monthly mean ± 1SE, Estradiol-17ß (E_2), Testosterone (T) and Progesterone (P) concentration in vitellogenic and non-reproductively active adult female *C. niloticus*. Sample sizes are shown above each column.

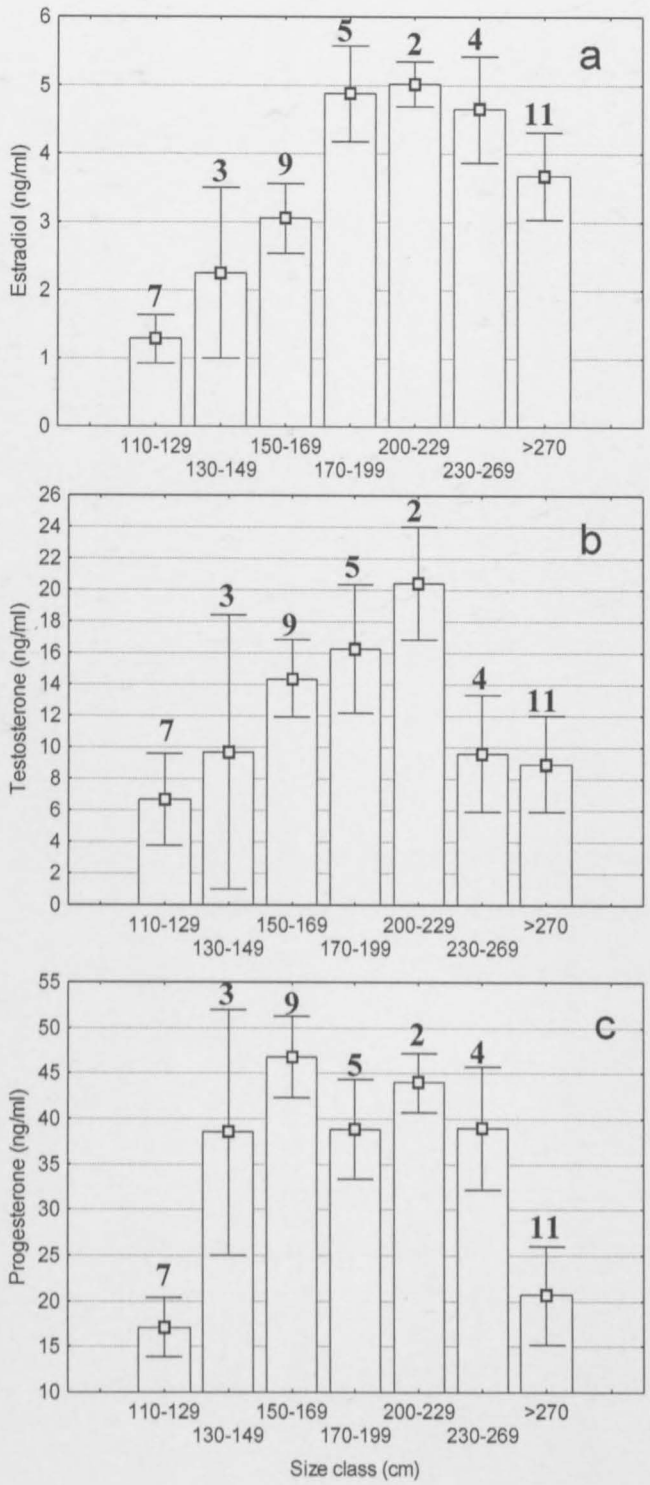

Figure 7. Mean ± 1SE Estradiol-17ß, Testosterone and Progesterone concentrations between June and September in female *C. niloticus* of different size classes (cm). Sample sizes are shown above each column.

2.3.4 Plasma lipids

Plasma lipid values in female Nile crocodiles showed considerable individual variation. There were no significant differences in triacylglycerol (TAG), cholesterol (CHO) or phospholipids (PL) concentrations between vitellogenic and non-reproductively active adult females ($p > 0.05$). Again, during the reproductive season from July to September, there were no significant differences in lipid concentrations between the different size classes (Figure 8 and Table 1).

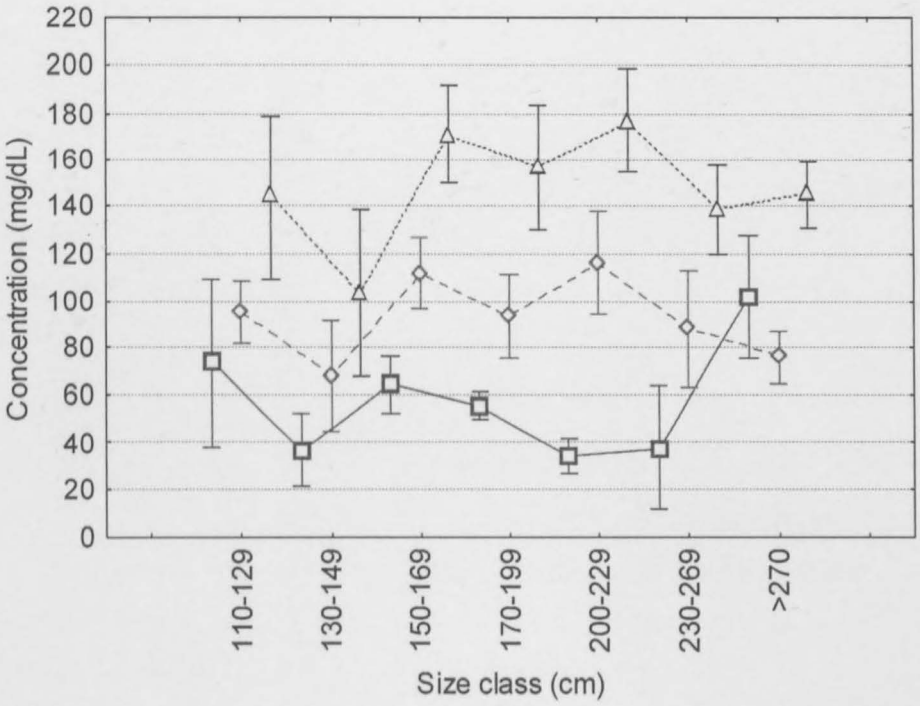

Figure 8. Mean ± 1SE triacylglycerol (open squares), total cholesterol (open lozenges) and phospholipid (open triangles) concentrations between June and September in female *C. niloticus* of different size classes (cm). See Table 1 for sample sizes.

Table 1. Mean ± SD triacylglycerol, total cholesterol and phospholipid concentrations between June and September in female *C. niloticus* of different size classes (cm).

Size class (cm)	N	Triacylglycerol Mean±SD (mg/dL)			Cholesterol Mean±SD (mg/dL)			Phospholipids Mean±SD (mg/dL)		
110-129	5	74.16	±	80.08	95.21	±	33.42	144.40	±	85.17
130-149	2	36.74	±	22.16	68.10	±	33.19	103.29	±	50.20
150-169	7	64.47	±	30.74	111.81	±	40.66	170.79	±	54.77
170-199	5	55.93	±	12.69	93.62	±	40.16	156.57	±	59.64
200-229	2	34.08	±	10.91	116.49	±	30.65	176.53	±	31.03
230-269	3	37.41	±	45.58	88.43	±	43.89	138.86	±	31.82
>270	10	101.68	±	82.56	75.80	±	35.42	145.09	±	45.11
All groups	**34**	**69.78**	**±**	**59.64**	**91.84**	**±**	**37.16**	**150.63**	**±**	**54.81**

Pearson's correlation analysis showed no relationship between sexual hormone concentrations and lipid concentrations, and there was no seasonally significant difference between summer and winter months ($p > 0.05$).

In addition, when analyzing the monthly variation in each lipid fraction for all size classes, no significant differences were noted throughout the year (Figure 9 and Table 2).

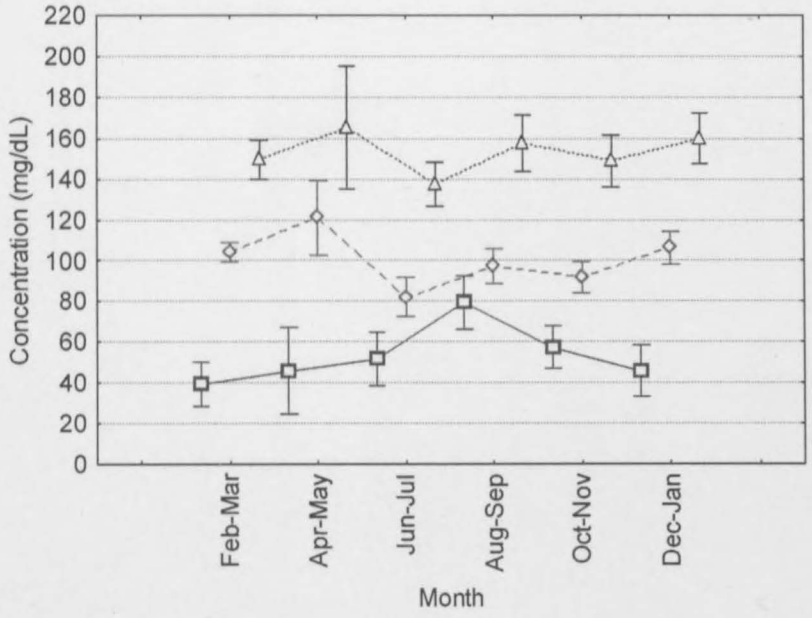

Figure 9. Mean ± 1SE triacylglycerol (open squares), total cholesterol (open lozenges) and phospholipid (open triangles) concentrations in female *C. niloticus*. See Table 2 for sample sizes throughout the year.

Table 2. Mean ± SD triacylglycerol, total cholesterol and phospholipid concentrations in female *C. niloticus* throughout the year.

Months	N	Triacylglycerol Mean±SD (mg/dL)	Cholesterol Mean±SD (mg/dL)	Phospholipids Mean±SD (mg/dL)
Feb-Mar	10	39.14 ± 34.20	104.49 ± 14.93	149.60 ± 29.97
Apr-May	3	45.77 ± 36.58	121.05 ± 31.24	165.35 ± 51.73
Jun-Jul	19	51.69 ± 54.68	82.07 ± 42.95	137.39 ± 45.89
Aug-Sep	21	79.74 ± 59.68	97.39 ± 40.15	157.59 ± 63.68
Oct-Nov	22	57.47 ± 50.27	92.04 ± 35.66	148.73 ± 59.89
Dec-Jan	13	45.91 ± 46.00	106.26 ± 30.22	159.54 ± 44.78
All groups	**88**	**57.41 ± 51.74**	**95.67 ± 36.37**	**150.66 ± 52.30**

2.4 DISCUSSION

Plasma collected from males of different sizes (110-270 cm TL) did not contain plasma vitellogenin detectable by electrophoresis, therefore electrophoresis was an effective means of determining which females were reproductively active (Guillette *et al.*, 1997). There seem to be no interferences from environmental contaminants which may induce production of vitellogenin in both sexes by the liver (Guillette *et al.*, 2000). Female *C. niloticus* in the Okavango Delta reach sexual maturity at approximately 232 cm TL which is similar to findings in *C. niloticus* in Uganda (Cott, 1961) and in Kenya (Graham, 1968), where more than 60 % of the females caught were sexually mature at 210 cm based on the size of the developing follicles. In Zimbabwe, female *C. niloticus* were found to start reproducing at approximately 262 cm (Kofron, 1990), which was determined by palpation of the abdomen in order to detect the presence of large ovarian follicles or oviductal eggs.

Contrary to what is observed on most crocodile farms, where females reproduce every year, only a portion of the wild adult females of the Okavango Delta population were reproductively active each season. This was estimated to be approximately 60 %. This also corroborates other similar studies on wild populations of crocodilians (Lance, 1989; Kofron, 1990; Guillette *et al.* 1997).

These data suggest that the Nile crocodile reproductive cycle in the Okavango Delta begins in June (winter) at the end of the rainy season when temperatures are low (Figure 2) and water levels start receding. Similar observations were reported for Nile crocodile in Zimbabwe (Kofron, 1990) and for the American alligator (Guillette *et al.*, 1997) where the onset of ovarian growth begins as water and air temperatures decline. Similar observations were also reported for the Saltwater crocodile, *C. porosus* (Webb and Manolis, 1989), but in addition, water levels seem be a factor controlling the onset of reproduction. The onset of ovarian activity occurs when plasma E_2 concentrations and electrophoretically detectable plasma Vtg levels rise. This pattern has also been noted in other reptiles such as alligators (Guillette *et al.*, 1997), turtles (Callard *et al.*, 1978; McPherson *et al.*, 1982; Strydom, 2001) and snakes (Bonnet *et al.*, 1994). Direct induction of Vtg in vitro has already established that E_2 is the sole inducer (Follett and Redshaw, 1974; Heck *et al.*, 1997; Herbst *et al.*, 2003) and its continuous presence is required for the maintenance of Vtg synthesis (Ho, 1987; Tata and Smith, 1979). The same pattern is observed for plasma testosterone concentrations which is understandable as T is a precursor of E_2 in the biosynthetic pathway. Alternatively, T is believed to have an inhibitory effect on vitellogenesis (Ho *et al*, 1981; Callard *et al.*, 1990).

Plasma progesterone concentration also followed the same pattern in vitellogenic females. The drop in P levels in August - September might permit oviposition as observed in some viviparous snake (Bonnet *et al.*, 2001) and oviparous lizards (Shanbhag *et al.*, 2001), where a drop in P concentration is believed to induce parturition/oviposition. Studies also showed that plasma P concentrations played an important role in the evolution of viviparity (Guillette, 1993). The variation of plasma sex steroid hormones observed in this study follows the same pattern as described for *C. niloticus* in Zimbabwe (Kofron, 1990), which share the same climate with Botswana, except that hormone levels are much higher in this study.

Contrary to findings in the American alligator, where estradiol-17ß (E_2) is low or non detectable during the height of the reproductive season in adult females showing no sign of reproductive activity (Guillette *et al.*, 1997), this study shows E_2 concentrations were comparable between reproductively and non-reproductively active females as well as for testosterone concentrations. Differences observed between reproductively and non-reproductively active females concerned the timing of the peaks for E_2 and T, which occurred earlier (June - July) in reproductively active females and progesterone concentrations were high from August to January in non-reproductively active females. Vitellogenic females displayed a drop in P levels after July.

The description of plasma sex hormone concentrations throughout the growth of female crocodiles gives us a better insight into the maturation process of crocodilians as well as providing baseline data. Studies on the American alligator (*Alligator mississippiensis*) showed that juvenile female alligators displayed a clear temporal pattern in plasma concentration of E_2 which reflected the same pattern in reproductively active female alligators but with concentrations being 7 to 16 times lower than in adult females prior to ovulation (Rooney *et al.* 2004). This analysis showed that plasma E_2 concentration increases as the animal grows and attains a maximum (± 4.5 ng/ml) once the animal reaches 170 cm TL, which is only 3 to 4 times higher than in juvenile female crocodiles. The same was observed with plasma T concentrations rising to 18 ng/ml, but when the animal reaches sexual maturity (230 cm TL), T levels decrease to ± 9 ng/ml. Plasma P concentrations have a different ontogenetic pattern. Small females have low P concentrations but P levels soon rise to ± 42 ng/ml and stay roughly constant throughout their growth. As the animals reach 270 cm TL, P concentration drops to similar levels as found in the small animals.

Plasma triacylglycerids, total cholesterol and phospholipids did not exhibit significant seasonal variation as observed in tortoises, *Gopherus agassizii* (Lance *et al*., 2002) and turtles such as *Chrysemys picta* (Duggan *et al*., 2001) or *Sternotherus odoratus* (McPherson and Marion, 1982). These studies used captive animals, therefore feeding was controlled year round. In this study, wild crocodiles were used and they have different feeding habits throughout the year. Juveniles and subadult crocodiles feed less during the winter months (June, July and August) than in the summer months (Wallace, 2006). Plasma lipid concentrations are known to increase during digestion, especially TAG (Norum 1992) and also depend on the lipid composition of the dietary item ingested. Therefore, we may not be able to correlate seasonal variation in plasma lipids and the reproductive cycle because of the interference caused by feeding regimes. It would be of great value to do further research on farmed crocodiles, where feeding is controlled.

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CHAPTER 3

MALE REPRODUCTIVE CYCLE OF THE NILE CROCODILE (*CROCODYLUS NILOTICUS*) IN THE OKAVANGO DELTA, BOTSWANA.

3.1 INTRODUCTION

Understanding reproductive patterns and their hormonal control in animals is indispensable for structuring proper management strategies for aquatic and terrestrial ecosystems, especially regarding key stone species such as crocodilians (Magnuson *et al.*, 1990).

Studies investigating physiological aspects of reproduction in crocodilians have focused primarily on the American alligator, *Alligator mississippiensis*, in which seasonal variation in plasma sex steroid concentrations of different populations was well studied (Lance, 1989; Guillette *et al.*, 1997; Rooney *et al.*, 2004), as well as the effect of environmental contaminants on hormone levels (Guillette *et al*, 1994; Guillette *et al*, 1996; Crain and Guillette, 1997; Guillette *et al.*, 2000). With regards to the Nile crocodile, *Crocodylus niloticus*, only one comprehensive study describing the reproductive cycle of a wild population in Zimbabwe has been published (Kofron, 1990). This study found that males reached sexual maturity around 270 cm in total length determined by the presence of live spermatozoa in the penile groove from May to September, when air temperatures were low. Mating occurred in July-August and was accompanied by high concentrations of testosterone.

In the last decade, reptiles have been used as biomonitors of endocrine-disrupting contaminants (EDCs) which may interfere with the normal functioning of the endocrine system (Guillette *et al*, 1995; Crain & Guillette, 1997). Crocodilians are long lived reptilians and are top predators in the aquatic environments they occupy, therefore, they are more susceptible to exposure to elevated levels of pesticides and other chemicals that bioaccumulate in food chains (Kidd *et al.*, 1998; Guillette and Iguchi, 2003). Comparative studies on pollution levels due to the use of dichloro-diphenyl-trichloroethane (DDT) for pest control as well as other pollutants, showed that fish and water in the Okavango Delta have low levels of DDT present when compared to nearby locations such as Lake Kariba in Zimbabwe, and also the lakes of temperate and sub arctic North America. (Mbongwe *et al*, 2003). Studying the reproductive cycle of the male Nile crocodile in its natural environment,

while the Okavango Delta is still in a so called pristine state will be useful as results could be used as a reference for comparative studies.

The objective of this study was to investigate changes in circulating testosterone levels during the natural reproductive cycle of male *C. niloticus* in the Okavango Delta, Botswana and to describe the change in plasma testosterone concentrations throughout their growth. In addition, we investigated the variation of plasma lipid components throughout the year and related these changes to seasonal changes in steroid hormone concentrations. A management plan is urgently required for the Okavango crocodile population and this study will provide essential baseline data regarding the reproductive endocrinology of wild Nile crocodiles in the Okavango Delta. This will in turn assist crocodile farmers, throughout Africa, with regards to improving productivity and therefore the sustainable utilization of crocodiles.

3.2 MATERIAL AND METHODS

3.2.1 Study area and Climate

The Okavango River is shared by three countries and has a catchment area of more than 300 000 km^2. The river rises in the Angolan Highlands as two tributaries, the Cubango and the Cuito rivers, and after several hundred kilometres, enters Botswana from the north where it forms a very broad floodplain known as the Panhandle, which eventually fans out to form the Okavango Delta (Figure 10). The Panhandle and its riverine floodplain, together with the upper part of the Delta, forms a permanent swamp while the lower parts of the Delta are seasonal floodplains. The size and timing of the annual flood depends more on rainfall in the catchment areas than it does on local rainfall. As soon as the Okavango River leaves the Panhandle area, it spreads out over the sands of the Kalahari forming a wide fan-shaped delta. The northern part of the Delta is characterized by shallow water, flooded grasslands, backwater swamps, ox-bow lakes and many hidden lagoons mostly interconnected by narrow waterways. Only a few main channels lined by tall reeds (mainly *Phragmites australis*), carry the remainder of the Okavango's water southwards through the Delta.

The climate of the area is sub-tropical with hot, rainy summers and warm, sunny and dry winters. Temperatures vary from an average maximum of 30-35° C from October to January to 7° C in the coldest months of June and July. The rainy season starts in November and ends in February. Another feature of the Okavango Delta is the flooding regime of the river. In the Panhandle region the floods start at the end of February and the water starts receding in June. The peak flows enter Botswana in April. The floodwater depends greatly on the Angolan catchments and local rainfall over the Delta (Figure 11 from Mendelsohn and El Obeid, 2004). During the height of the flooding season, crocodiles disperse into the floodplains, probably to follow the fish and find dry ground for basking during the cold winter months, which makes it difficult and often impossible to capture them using our current capture techniques (pers. comm. A. J. Leslie).

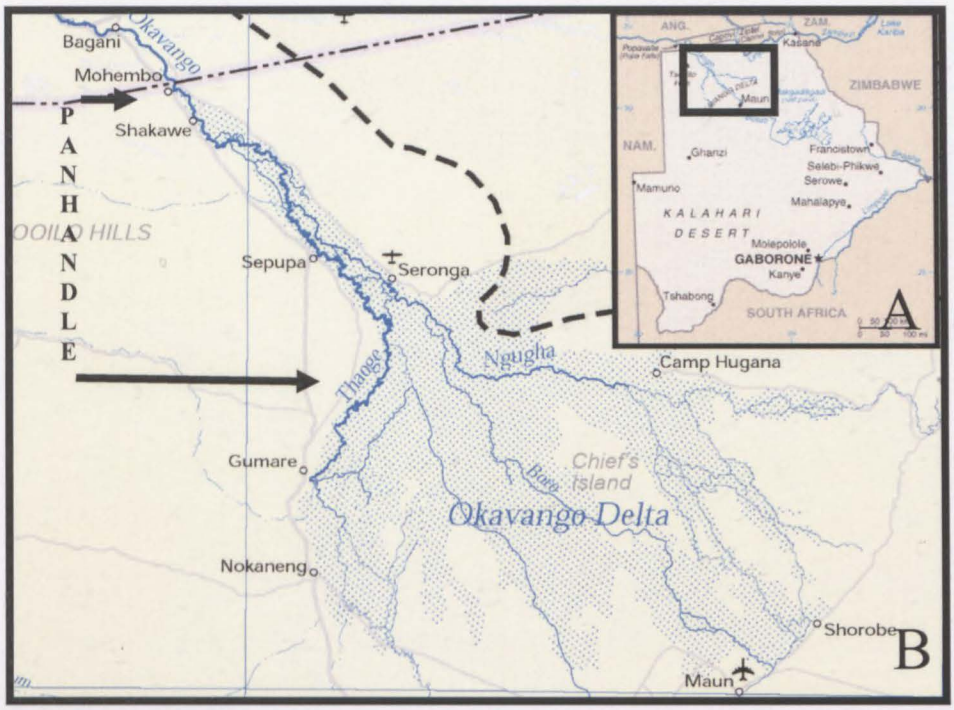

Figure 10. The Okavango Delta is situated in the north west of Botswana (A). Nile crocodiles were captured in the Panhandle area (B). Adapted from CIA World Factbook.

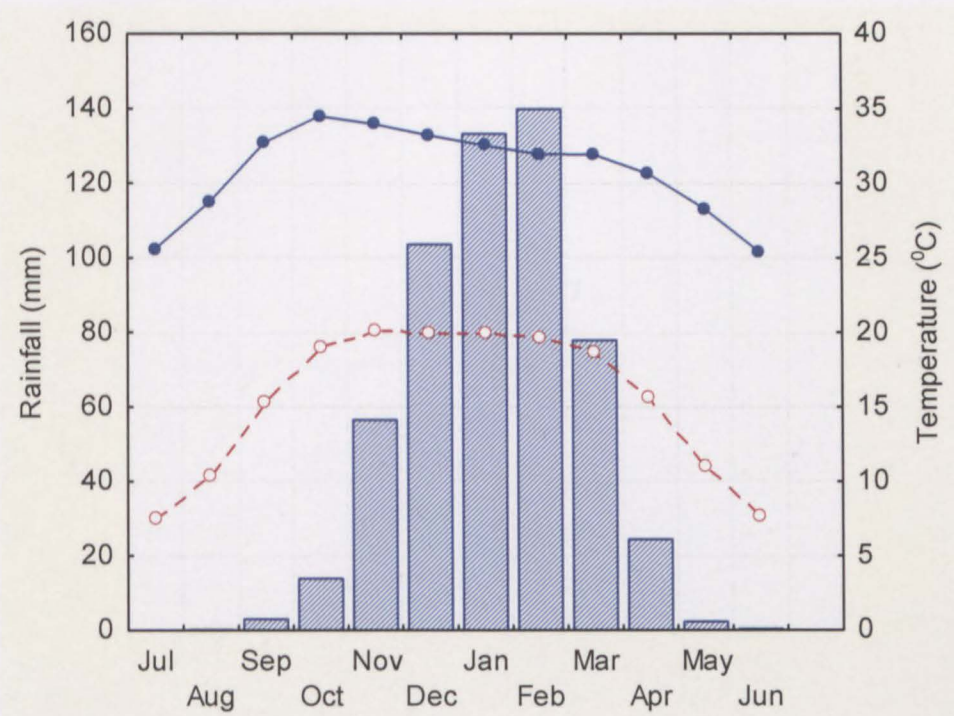

Figure 11. Total monthly rainfall (bar graph), maximum ambient air temperatures (closed circles) and minimum ambient air temperatures (open circles), recorded at Shakawe, Botswana.

3.2.2 Study organism

Crocodylus niloticus (Laurenti, 1768) belongs to the class Reptilia, order Crocodilia, suborder Eusuchia, family crocodylidae, and subfamily Crocodylinae. Crocodilians belong to the group of *archosaurs* ("ruling reptiles") which also included the extinct thecodonts, its earliest and most primitive members, the dinosaurs, the pterosaurs or flying reptiles (Benton, 1982). Crocodilians of the suborder Eusuchia appeared in the Cretaceous period (140 to 65 million years ago) and all living crocodilians belong to this suborder. They occupy an important position in the evolution of vertebrates as they exhibit anatomical features found only in dinosaurs and features common to birds and mammals (Brochu, 2001). Today there is 23 species of crocodilians all located between the latitudes of cancer (23.5° north) and Capricorn (23.5° south). The Nile crocodile is one of three species living on the African continent, the two other species being the dwarf crocodile (*Osteolaemus tetraspis*) and the long-snouted crocodile (*Crocodylus cataphractus*).

The Nile crocodile is unevenly distributed throughout the Okavango system, although the majority of the breeding population is found in the Panhandle, where permanent water is available.

3.2.3 Capture methods

Two capture methods were used: (i) At night, using a 4.8 meter flat bottomed aluminium boat propelled by a 60 hp engine, crocodiles were located using a 500 000 candle light power spot light which, once shone into the crocodile's eyes, reflected back a red glow due to the presence of a retinal tapetum. Once spotted, the beam of light remained on the crocodile's eyes so as to mesmerize it, making it possible to approach the animal by boat. Crocodiles estimated to be smaller than 120 cm were captured by hand. Crocodiles between 120 and 230 cm were captured using a swivelling noose (Animal Handling Co., South Africa) which was placed over the snout and pulled tight in the neck region. Crocodiles were then brought onto the boat, jaws were taped shut and the animals were physically constrained. Animals larger than 230 cm, were captured using a noose attached to a climbing rope, which was secured to the boat. The crocodile was allowed to swim so as to tire it out before it was brought onto the boat. (ii) During summer months, baited box and Pitman traps were strategically placed on river banks. Traps were baited at sunset and checked at first light the next morning. Captured animals were immediately restrained and the necessary data collected. No chemical mobilization took place.

3.2.4 Sampling techniques

Crocodiles were captured in the Panhandle area (Figure 10) every month between January 2002 and August 2005, except for the month of May when flooding was at a peak and crocodiles migrated into the flood plains making capture difficult. A 0.5 - 5.0 ml blood sample was drawn between 3 and 10 min after capture from the caudal vein in the tail (Gorzula *et al.*, 1976; Leslie, 1997). Once all the data were collected the crocodile was released at the site of capture. Blood was transferred to microcentrifuge tubes (Ependorff, Germany), placed on ice, and later when back in the field laboratory, centrifuged for 5 minutes at approximately 1500 x *g*. The plasma supernatant was aspirated, frozen at -15° C and stored at -80° C until further analysis in South Africa. Once back in the laboratory at Stellenbosch University all samples were again centrifuged with a UniEquip (Martinsred, Germany) bench top centrifuge at 4° C for 2 min at 5000 x *g* before analysis.

3.2.5 Sperm sample and size at sexual maturity

Total length (TL) and snout-to-vent length (SVL) of all captured crocodiles was recorded using a flexible measuring tape (±1 mm). In order to determine the size at which male Nile crocodiles reach sexual maturity, sperm samples were collected from animals with a TL greater than 180 cm. The penis was extruded from the cloaca by manual pressure (Guillette *et al.* 1996). Sperm was collected along the penal groove using a spatula, and was then smeared onto a slide and fixed in 95 % ethanol. The sperm smear was stained with Gill's haematoxylin and Yellow Eosin. The sperm smears were observed using a microscope (x1000) and the presence or absence of spermatozoa was recorded. Males with spermatozoa present in the semen were considered sexually mature. Therefore, the size of the smallest male producing spermatozoa was considered to be the size at which the Okavango's male Nile crocodiles attain sexual maturity.

3.2.6 Plasma Testosterone analysis

Plasma testosterone (T) concentrations were measured using commercial enzyme immunoassay (EIA) test kits (International Immuno-Diagnostics, California, USA). The antibodies used were highly specific with very little cross-reactivity to other steroids (less than 2 %). Validation of the assays for crocodile plasma revealed an intra-assay coefficient of variance of less than 6 % and an inter-assay variation of less than 9 %. Plasma samples diluted with buffer solution showed good parallelism when the standard curves were prepared using the steroid samples supplied.

Tests were performed in duplicate according to the manufacturer's protocol. In brief, 10 µl of standard or plasma (diluted with T-HRP conjugate) were dispensed into each Goat Antirabbit IgG-coated microtiter well. One hundred µl T-HRP conjugate and 50 µl of Rabbit anti-T reagent were added to each well and mixed. Plates were incubated at 36° C for 75 minutes. The plates were rinsed five times with distilled water. One hundred µl of substrate (tetra-methyl benzidine) was then added to each well and incubated at room temperature for 15 minutes. The reaction was stopped with 50 µl 1 N HCl. Absorbance was measured at 450 nm, using a microtiter plate reader (Power Wave x, Bio-Tek instruments, INC).

3.2.7 Lipid analysis

Triglyceride concentrations were determined using a colorimetric enzyme assay (Triglycerides MR, Linear Chemicals, Spain). The assay was performed following the manufacturer's protocol using ab-well microtiter plates, each containing 1 blank (300 µl), 1 standard (303 µl) and 27 samples (303 µl). Samples and standards were tested in duplicate. Absorbance was measured at 500 nm using a microtiter plate reader (Power Wave x, Bio-Tek Instruments, INC). Total Cholesterol (CHO) and Phospholipid (PL) concentrations were also determined using a colorimetric enzyme assay (Cholesterol MR, Linear Chemicals, Spain and Phospholipids, CHO-POD; Spinreact, Spain) in the same manner as for the Triglycerides and absorbances were measured at 550 nm and 505 nm, respectively.

3.2.8 Statistical analysis

Differences in plasma testosterone and lipid concentrations between months or size classes were tested using one-way ANOVA or Student t-test (Statistica 7, StatSoft, Inc. 1984/2004). Data sets were tested for normality prior to statistical analyses and were log transformed prior to testing if heterogeneity was detected. Correlations between plasma lipids and steroid hormone concentrations were determined using a Pearson's correlation analysis.

3.3 RESULTS

3.3.1 Sperm sample and size at sexual maturity

Seventeen sperm samples were collected throughout the year from crocodiles ranging from 187 cm to 471 cm. Spermatozoa were present in the crocodile's semen from June to the end of October in 9 of the 11 samples collected during that period (Figure 12 and 13). During those months, the smallest crocodile which had spermatozoa present was 197 cm TL and the other 8 samples were from crocodiles with a TL greater than 270 cm.

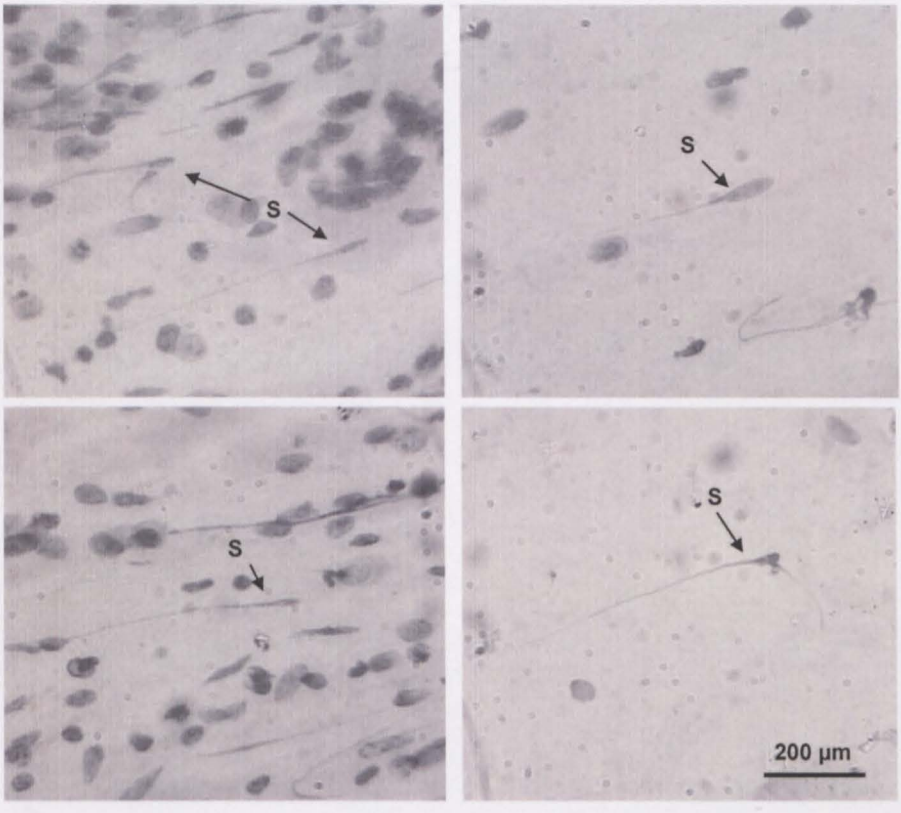

Figure 12. Sperm smears collected from 4 different adult male crocodiles producing spermatozoa (s).

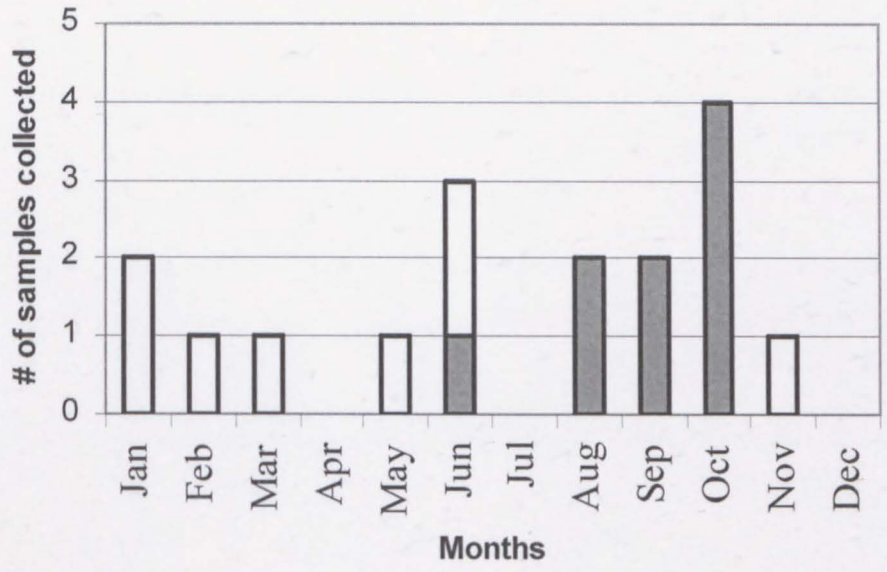

Figure 13. The number of sperm samples collected throughout the year. Grey bars: samples with spermatozoa present. White bars: samples without spermatozoa present.

Unfortunately no samples were collected from animals measuring between 197 and 270 cm TL during the months when spermatozoa were present in the semen. However, we assumed that male crocodiles reached sexual maturity at 197 cm TL. Animals over 270 cm TL were also sampled in January, February and March and no spermatozoa were observed in the semen samples.

3.3.2 Plasma testosterone in adult males.

No significant variations in plasma T concentrations were detected between months (Figure 14). Plasma T concentrations varied greatly within each month (Table 3).

3.3.3 Variation in plasma testosterone concentrations throughout growth

There was no significant variation in monthly plasma T concentrations in any given size class or between size classes ($p > 0.05$). There was considerable individual variation in animals smaller then 180 cm in TL and no clear seasonal pattern in plasma testosterone concentrations was observed. Larger crocodiles displayed a seasonal pattern. Animals between 180 and 269 cm in total length had high testosterone concentrations in October - November whereas older animals with a TL greater than 269 cm had elevated plasma testosterone levels in August - September (Figure 15).

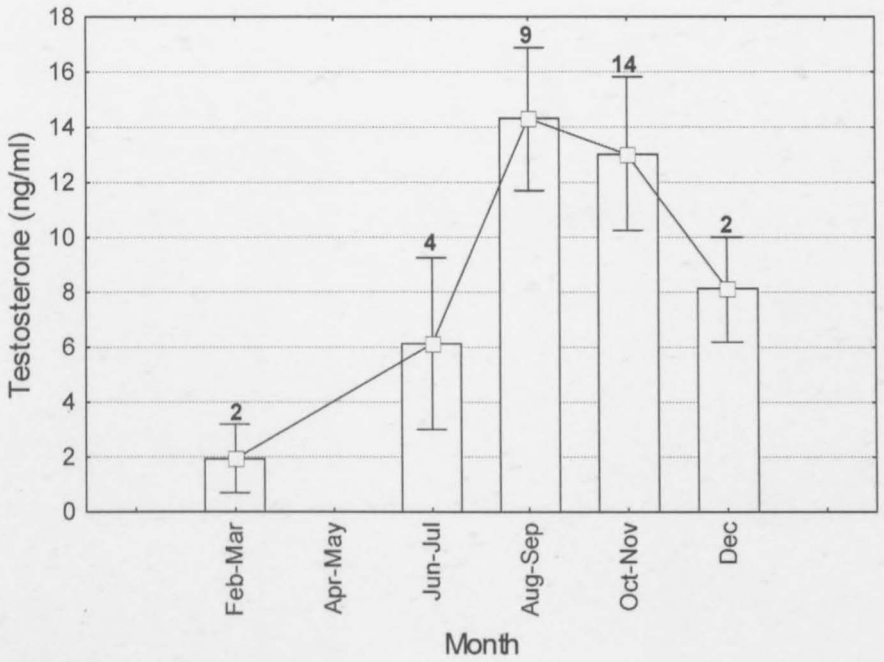

Figure 14. Monthly mean ± 1 SE testosterone in adult male *C. niloticus*. Sample sizes are indicated above each column.

Table 3. Mean ± 1 SE plasma testosterone concentrations in adult male *C. niloticus*.

Months	N	Testosterone (ng/ml) Means±SD (ng/ml)		
Feb	1	3.20	±	0.00
Mar	1	0.70	±	0.00
Jun	2	0.85	±	0.07
Jul	2	11.40	±	1.98
Aug	4	18.25	±	10.24
Sep	5	11.12	±	3.60
Oct	5	10.16	±	8.96
Nov	9	14.62	±	11.33
Dec	2	8.10	±	2.69
All Groups	31	11.47	±	9.02

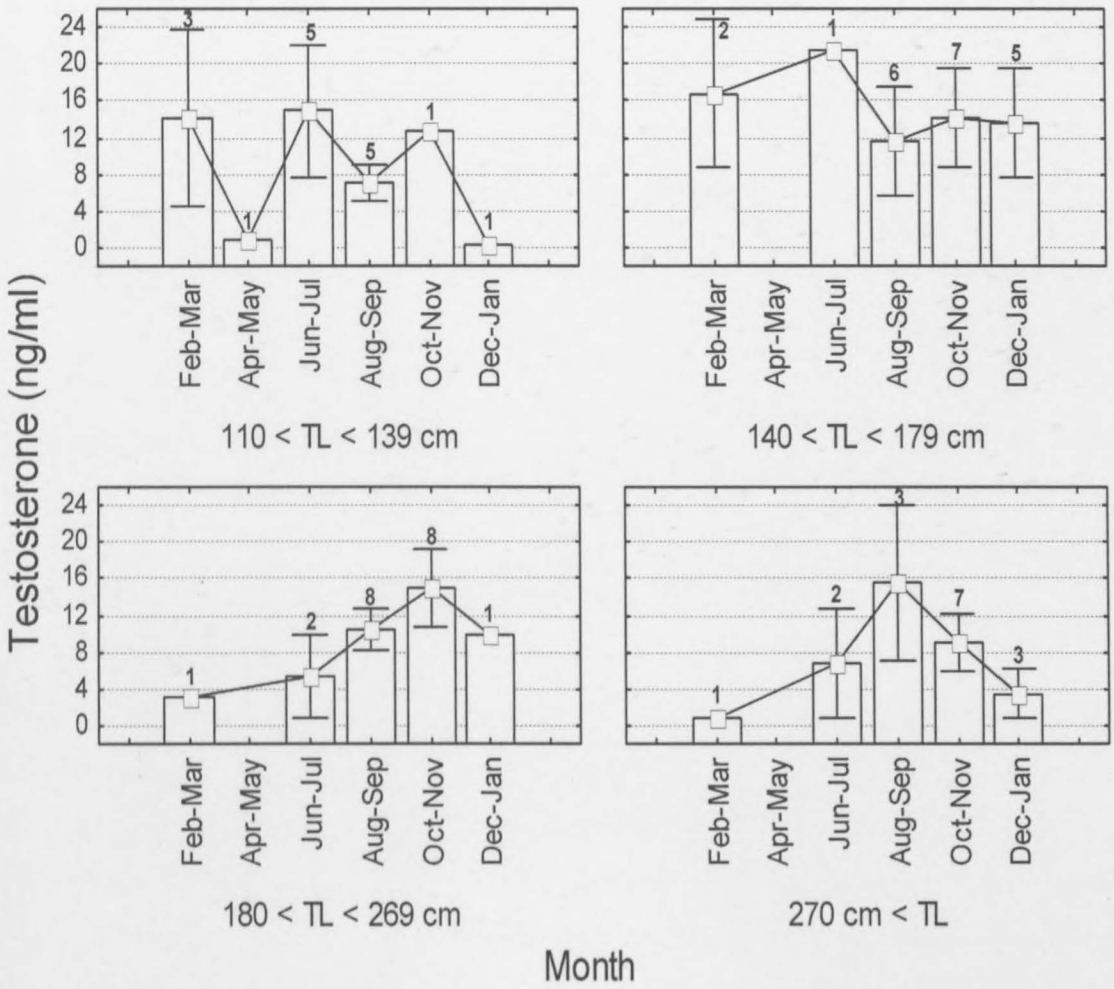

Figure 15. Monthly mean ± 1 SE testosterone concentrations in male *C. niloticus* of different size classes (cm). Sample sizes are indicated above each column.

3.3.4 Plasma lipids

There were no significant differences in triacylglycerol (TAG), cholesterol (CHO) or phospholipid (PL) concentrations in adult males (TL > 197 cm. Figure 16 and Table 4).

During the reproductive season from June to September, when adult males produce spermatozoa, there were no significant differences in lipid concentration between the different size classes (Figure 17 and Table 5).

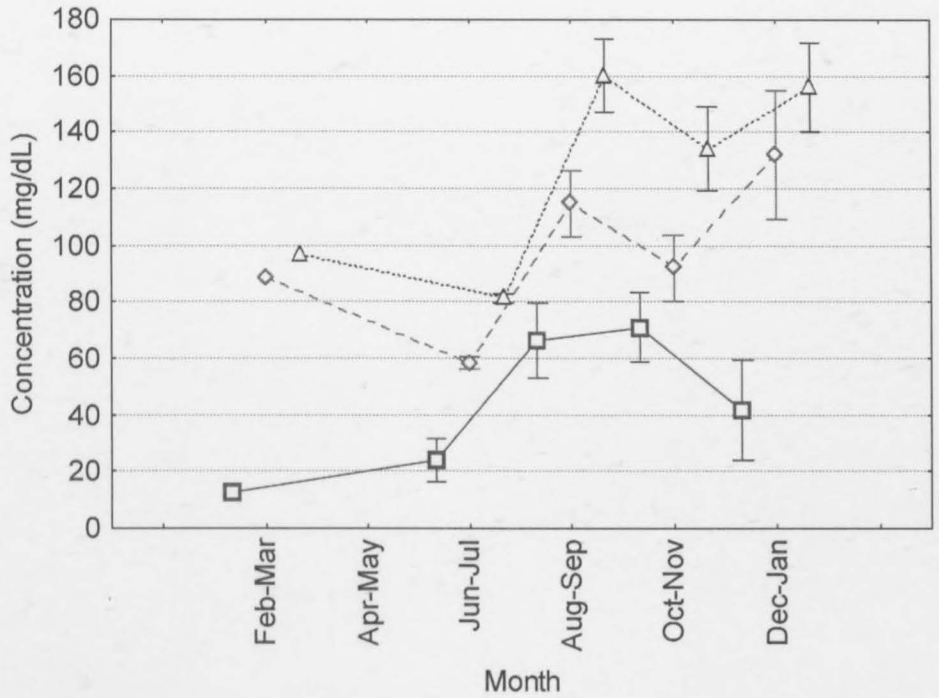

Figure 16. Monthly mean ± 1SE triacylglycerol (open squares), total cholesterol (open lozenges) and phospholipid (open triangles) concentrations in adult male *C. niloticus*. See Table 2 for sample sizes.

Table 4. Mean ± SD triacylglycerol, total cholesterol and phospholipid concentrations in adult males *C. niloticus* throughout the year.

Months	N	Triacylglycerol Mean±SD (mg/dL)			Cholesterol (mg/dL)	Mean±SD		Phospholipids (mg/dL)	Mean±SD	
Feb-Mar	1	12.34568			88.2353			96.5625		
Jun-Jul	11	24.19216	±	13.20806	58.3491	±	3.59334	81.2007	±	3.23763
Aug-Sep	15	66.19035	±	44.37259	114.7958	±	38.35688	159.8714	±	43.06279
Oct-Nov	4	71.04521	±	48.73494	92.0260	±	44.97879	134.0942	±	55.10484
Dec-Jan	34	41.46091	±	35.39633	132.2197	±	45.50565	155.9375	±	27.00550
All Grps	88	60.13346	±	44.98868	101.0384	±	43.37795	138.8713	±	49.93890

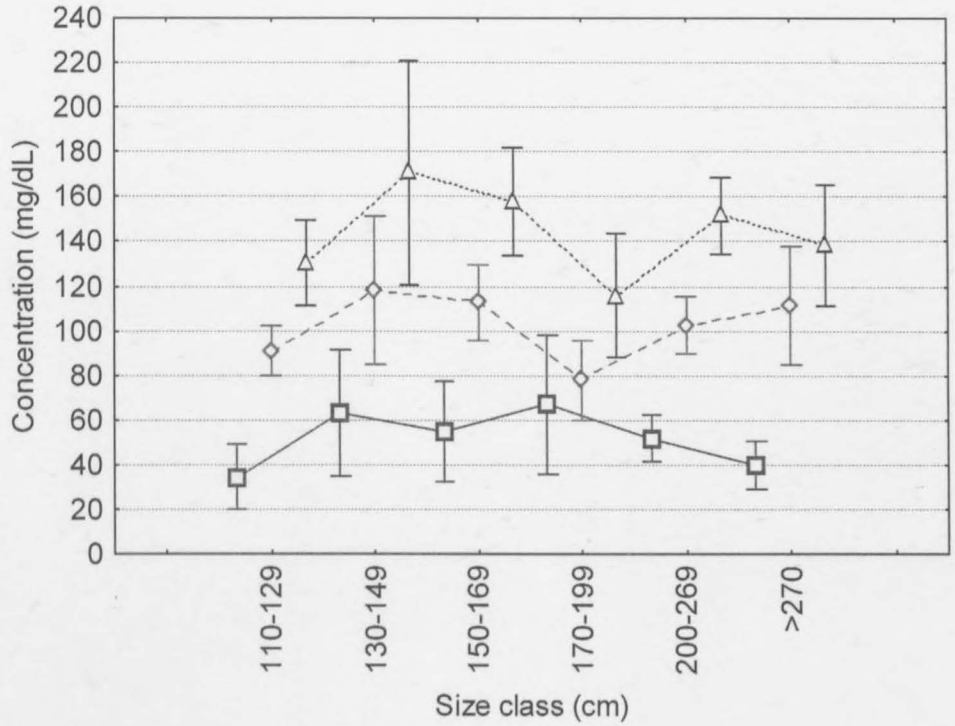

Figure 17. Mean ± 1SE triacylglycerol (open squares), total cholesterol (open lozenges) and phospholipid (open triangles) concentrations during the reproductive season (June - September) in male *C. niloticus* of different size classes (cm). See Table 3 for sample sizes.

Table 5. Mean ± SD triacylglycerol, total cholesterol and phospholipid concentrations during the reproductive season (June-September) in male *C. niloticus* of different size classes throughout the year.

Size class (cm)	N	Triacylglycerol Mean±SD (mg/dL)	Cholesterol Mean±SD (mg/dL)	Phospholipids Mean±SD (mg/dL)
110-129	7	34.26608 ± 38.45816	90.8478 ± 29.41281	130.2632 ± 50.8423
130-149	4	63.24615 ± 55.96502	118.1362 ± 66.54619	170.8100 ± 100.5050
150-169	4	54.78872 ± 44.60353	112.5892 ± 33.89353	157.6809 ± 48.3302
170-199	5	66.96788 ± 68.90520	77.7030 ± 39.32731	115.8849 ± 62.2621
200-269	6	51.61498 ± 25.64661	102.5067 ± 31.33138	151.7325 ± 41.4177
>270	5	40.03479 ± 24.16125	111.1969 ± 58.80084	138.4112 ± 60.3335
All Grps	31	50.21630 ± 42.16282	100.5928 ± 41.84200	142.1833 ± 57.9581

There was no significant correlation between plasma testosterone concentrations and lipid concentrations (Pearson's Correlation Coefficient; $p > 0.05$) and no significant differences where noted between summer and winter months ($P > 0.05$).

In addition, when analyzing the monthly variation of each lipid for all size classes, no significant differences were noted (Figure 18 and Table 6) throughout the year.

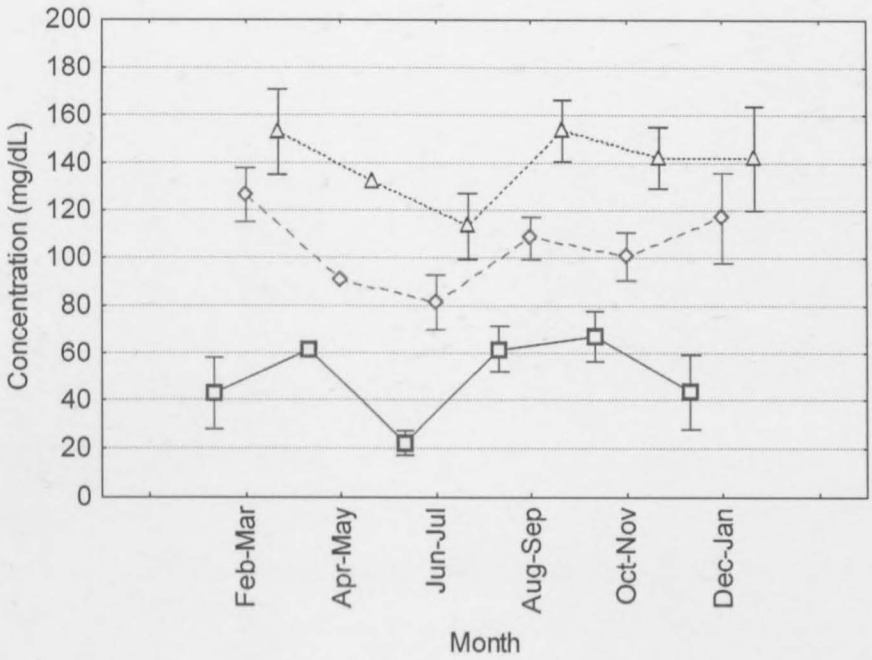

Figure 18. Monthly mean ± 1SE triacylglycerol (open squares), total cholesterol (open lozenges) and phospholipid (open triangles) concentrations in male *C. niloticus*. See Table 4 for sample sizes.

Table 6. Mean ± SD triacylglycerol, total cholesterol and phospholipid concentrations in male *C. niloticus* throughout the year.

Months	N	Triacylglycerol Mean±SD (mg/dL)	Cholesterol Mean±SD (mg/dL)	Phospholipids Mean±SD (mg/dL)
Feb-Mar	6	42.83088 ± 36.18073	126.4576 ± 28.16307	152.9331 ± 43.38729
Apr-May	1	61.16838 ± 0.00000	90.7438 ± 0.00000	131.9079 ± 0.00000
Jun-Jul	9	22.10815 ± 14.22143	81.6629 ± 34.44453	113.5234 ± 41.92648
Aug-Sep	22	61.71509 ± 44.55794	108.3368 ± 42.81008	153.9077 ± 60.27519
Oct-Nov	23	67.11988 ± 51.95032	100.6251 ± 46.79153	142.2339 ± 61.38780
Dec-Jan	10	43.46528 ± 49.69205	116.8647 ± 59.75562	142.0139 ± 65.38799
All Grps	71	54.27139 ± 45.94744	104.9421 ± 45.07271	142.9631 ± 57.43108

3.4 DISCUSSION

In this study, one sample collected from a male of approximately 200 cm in TL had fully formed spermatozoa in the semen collected from the penal groove. Unfortunately all other samples were collected from animals larger than 270 cm in TL. This suggests that males reach sexual maturity around 200 cm in TL, but it is unlikely that they succeed in breeding due to competition with larger animals for access to females during the mating season (Modha, 1967, Joanen & Mcnease, 1980). This corroborates results from a study on Nile crocodiles (*Crocodylus niloticus*) in Kenya, where the smallest mature male was 212 cm in TL (Graham, 1968). In Zambia, male crocodiles become sexually mature at 230 cm in TL (Cott, 1961) and around 270 cm in Zimbabwe (Kofron, 1990).

Detection of spermatozoa in the semen samples started in June, during the dry season when air temperatures were low, and ceased at the end of October at the start of the rainy season in summer. Nile crocodiles in Zimbabwe also produced spermatozoa during the same months when air temperatures were low (Kofron, 1990) as did American alligators, *Alligator mississippiensis* (Lance 1989). During the same period, elevated plasma testosterone concentrations were noted and peaked in August (18.25 ± 10.24 ng/ml) during the mating season. Elevation of plasma T during the mating season is commonly observed in vertebrate taxa, since T promotes mating and male agonistic behaviours (Lindzey and Crews, 1986; Schuett, 1994; Schuett *et al*. 1997).

The description of plasma sex hormone concentrations throughout the growth of male crocodiles provides a better understanding of the maturation process in crocodilians, as well as much needed baseline data. Studies of the American alligator (*A. mississippiensis*) showed that juvenile male alligators displayed a clear, temporal pattern of plasma testosterone concentrations which reflected the same pattern as sexually mature male alligators but with concentrations being 1/10 to 1/100 of adult males (Rooney *et al*. 2004). In this study, concentrations were comparable between immature and mature crocodiles. There was individual variation in plasma testosterone concentrations in animals measuring 110 - 139 cm TL and no clear pattern could be identified. Immature crocodiles above 140 cm in TL had elevated plasma testosterone concentrations throughout the year. Only when male crocodiles reached 180 cm in total length was a clear seasonal pattern observed.

Total cholesterol and phospholipids did not exhibit significant seasonal variation as observed in tortoises, *Gopherus agassizii* (Lance *et al*., 2002) and turtles such as *Chrysemys picta* (Duggan *et al*., 2001) or *Sternotherus odoratus* (McPherson and Marion, 1982). In adult males, plasma triacylglycerol (TAG) concentrations followed the same general pattern as plasma T concentrations, rising in June - July, through October - November and then gradually declining. However, because plasma lipid concentrations are known to increase during digestion, in particularly TAG (Norum 1992), and concentrations also depend on the lipid composition of the dietary item ingested, we may not be able to correlate seasonal variation in plasma lipids and the reproductive cycle because of the interference caused by feeding regimes. Previous studies on plasma lipids used captive animals, therefore, feeding was controlled year round, whereas we dealt with wild crocodiles which have different feeding habits during the year. Juvenile and subadult crocodiles feed less during the winter months (June, July and August) than during the summer months (Wallace, 2006) and most certainly adult crocodiles as well. It would be of great value to do further research on farmed crocodiles, where feeding is controlled.

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CHAPTER 4

SEXUAL SIZE DIMORPHISM IN THE NILE CROCODILE, (*CROCODYLUS NILOTICUS*) IN THE OKAVANGO DELTA, BOTSWANA.

4.1 INTRODUCTION

Sexual dimorphism is defined as a morphological differentiation of sexually mature males and females. In most animal species, males and females differ significantly in body size as well as in relative dimensions of certain corresponding body parts, a phenomenon referred to as sexual size dimorphism (SSD). Size divergence is one of the most conspicuous differences between males and females, and such dimorphism has been intensively studied in a wide range of animals (Darwin 1871, Bristowe 1929, Pietsch 1976, Clutton-Brock *et al.* 1977, Berry and Shine 1980, Shine 1989, 1994; Anderson 1994; Vollrath 1998).

Research has focused on the ultimate or adaptive causes of SSD (Carothers 1984, Price 1984, Arak 1988, Shine 1989, Anderson and Vitt 1990, Fairbairn 1997, amongst others) as well as proximate causation of SSD, whether adaptative or not (Cheverud *et al.* 1985, Shine 1986, 1989, 1990, Anderson and Vitt 1990, Fairbairn 1990, Stamps 1993, Andrew and Stamps 1994, Watkins 1996, Beaupre *et al.* 1998). Processes usually invoked as causal mechanisms for SSD in animals are: sexual selection, natural selection, intersexual differences in growth patterns, accidental hormonal effects during ontogeny and phyllogenetic conservatism (Camilleri and Shine, 1990; Perez-Mellado and De La Riva, 1993; Shine, 1994; Jennsen *et al.*, 1995; Bull and Pamula, 1996; Hews, 1996). Sexual size dimorphism can obviously also evolve through the combined effects of these factors acting either collectively or in sequence (Shine, 1989).

The evolution of SSD requires that selection acts differently on male and female body sizes (Parker 1992). Female fecundity and reproductive success increase with increasing body size, referred to as fecundity selection (Darwin 1871; Shine and Schwartzkopf 1992). When males are the larger sex, SSD has been attributed mainly to sexual selection (Borgia 1981; Gwynne 1982; Hedrich and Terneles 1989, Moller 1990; Andersson 1994), resulting either from mate choice (intersexual selection) or from direct competition between individuals of the same sex, namely, intrasexual selection (Darwin 1871). If a large male can monopolize more females than a smaller male can, then the male's reproductive success may rise with increasing body

size, even more strongly than a female's reproductive success does (Trillmich and Trillmich 1984; Saether *et al.* 1986; Shine 1994).

Many factors may affect the direction and the intensity of SSD. For example: size at birth, juvenile and adult growth rates, age and size at sexual maturity and survival. Each of these traits may be a different target of natural selection and therefore SSD is a central issue in evolutionary biology. The actual SSD observed in a given population results from a combination of these factors, and there are myriad different possible scenarios (Stamps and Krishman 1997).

Environmental fluctuations can also influence growth rate and survival, further complicating the situation (Shine 1990; Berrigan and Charnot 1994). Overall, the respective influences of different selective forces (e.g., sexual selection, selection for fecundity, ecological selection) on each sex are often inextricably mixed. For example: in species in which juvenile and adult phases differ considerably in morphology and ecology, such as in holometabolous insects. Although interesting, these life-history patterns certainly complicate SSD analysis and it may be easier to study a species with direct development. Focusing on species with little or no sexual divergence in ecology (e.g. feeding habits, activity period, predation pressures) or morphology (e.g. extravagant characters, colours), we can remove most of the confounding effects due to ecological differences and allometry (Hill 1950; Gould 1966, 1974; McGowan 1994; Fairbairn 1997; Bonnet *et al.* 1998, 2001). Additionally, studying long-lived species increases statistical power for evaluating relationships among factors influencing growth and adult body size (through trade-offs amongst growth, age and size at maturity, reproduction and survival) because long-lived species often exhibit considerable inter-individual variation in growth rate and maturation patterns (Madsen and Shine 2000). This is why the Nile crocodile, *Crocodylus niloticus,* is a well suited species for understanding SSD. The aim of this chapter is therefore to provide baseline information on growth and SSD in *C. niloticus*, in Botswana.

4.2 MATERIALS AND METHODS

4.2.1 Study area and Climate

The Nile crocodile is unevenly distributed throughout the Okavango system, although the majority of the breeding population is found in the Panhandle, where permanent water is available. In this study all crocodiles were captured in the Panhandle area (Figure 1).

The climate of the area is sub-tropical with hot, rainy summers and warm, sunny and dry winters. Temperatures vary from an average maximum of 30-35° C from October to January to 7° C in the coldest months of June and July. The rainy season starts in November and ends in February (Mendelsohn and El Obeid, 2004. Another feature of the Okavango Delta is the flooding regime of the river. In the Panhandle region the floods start at the end of February and the water starts receding in June. The peak flows enter Botswana in April. The floodwater depends greatly on the rainfall in the Angolan catchments and local rainfall over the Delta (Mendelsohn and El Obeid, 2004). During the height of the flood season, crocodiles disperse into the floodplains, probably to follow the fish and to find dry ground for basking during the cold winter months. This makes it difficult or even impossible to collect data during these months using current capture techniques.

4.2.2 Study organism

Crocodylus niloticus (Laurenti, 1768) belongs to the class Reptilia, order Crocodilia, suborder Eusuchia, family crocodylidae, and subfamily Crocodylinae. Crocodilians belong to the group of *archosaurs* ("ruling reptiles") which also included the extinct thecodonts, its earliest and most primitive members, the dinosaurs, the pterosaurs or flying reptiles (Benton, 1982). Crocodilians of the suborder Eusuchia appeared in the Cretaceous period (140 to 65 million years ago) and all living crocodilians belong to this suborder. They occupy an important position in the evolution of vertebrates as they exhibit anatomical features found only in dinosaurs and features common to birds and mammals (Brochu, 2001). Today there is 23 species of crocodilians all located between the latitudes of cancer (23.5° north) and Capricorn (23.5° south). The Nile crocodile is one of three species living on the African continent, the two other species being the dwarf crocodile (*Osteolaemus tetraspis*) and the long-snouted crocodile (*Crocodylus cataphractus*).

4.2.3 Capture methods

Two capture methods were used: (i) At night, using a 4.8 meter flat bottomed aluminium boat propelled by a 60 hp engine, crocodiles were located using a 500 000 candle light power spot light which, once shone into the crocodile's eyes, reflected back a red glow due to the presence of a retinal tapetum. Once spotted, the beam of light remained on the crocodile's eyes so as to mesmerize it, making it possible to approach the animal by boat. Crocodiles estimated to be smaller than 120 cm were captured by hand. Crocodiles between 120 and 230 cm TL were captured using a swivelling noose (Animal Handling Co., South Africa) which was placed over the snout and pulled tight in the neck region. Crocodiles were then brought onto the boat, jaws were taped shut and the animals were physically constrained. Animals larger than 230 cm, were captured using a noose attached to a climbing rope, which was secured to the boat. The crocodile was allowed to swim so as to tire it out before it was brought onto the boat. (ii) During summer months, baited box and Pitman traps were strategically placed on river banks. Traps were baited at sunset and checked at first light the next morning. Captured animals were immediately restrained and the necessary data collected. No chemical mobilization took place.

4.2.4 Sampling procedures

Crocodiles were captured every month between January 2002 and August 2005, except for the month of May when the river flooding was at a peak and crocodiles migrated into the flood plains which made capture difficult. Seven traits were measured on all captured crocodiles (Figure 19): Total length (TL), snout-to-vent length (SVL), neck circumference (NC), base of tail circumference (BTC), head length (HL), head width (HW) and head depth (HD). These measurements were used to calculate tail length (TL-SVL) and trunk length (SVL-HL) as well as several measures of head and body morphology relative to body size or head size (TL/SVL, Trunk/SVL, Tail/SVL, HL/SVL, HW/SVL, HD/ SVL, HW/HL and HD/HL). Snout-vent length was chosen as the basic index of body size because TL was dependent on tail length, the tip of which was sometimes damaged. All body measurements were recorded to the nearest 1.0 mm using a flexible tape and all head measurements were recorded to the nearest 0.1 mm using a pair of callipers. Sex was determined by examination of the cliteropenis of animals > 60 cm in TL, as the application of this method in hatchlings and young generally leads to a high rate of misclassification (Allstead & Lang, 1995). Crocodiles < 60 cm TL were sexed as "unknown". Female *C. niloticus* reach sexual maturity at 230 cm TL (127 cm SVL), whereas males reach sexual maturity at 180 cm TL, but are probably not reproducing due to

competition with bigger males (Chapter 3). For the purpose of this study, males were considered to be "adult" animals if they were > 230 cm TL (127 cm SVL). Therefore, three different size classes were tested for SSD: (i) juveniles (28<SVL<72 cm), (ii) sub-adults (72<SVL<127 cm) and (iii) adults (127 cm <SVL).

4.2.5 Statistical analysis

Morphometrical variables (except ratios) were log-transformed prior to analysis to meet the assumptions of the statistical tests. Unpaired two-tailed t-tests and analysis of covariance (ANCOVA) were used to test for sexual size dimorphism (Statistica 7, StatSoft Inc.).

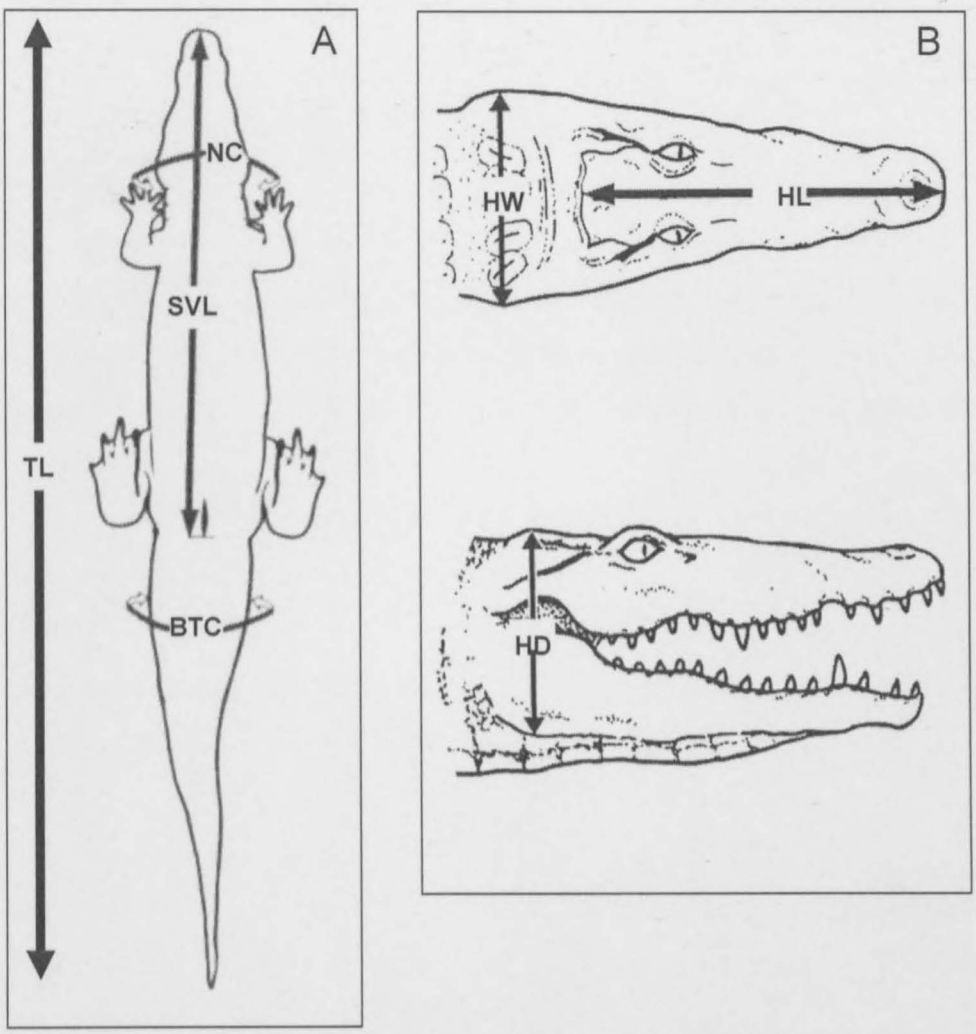

Figure 19. Schematic diagram of measurements recorded for (A) body and (B) head. TL = total length, SVL = snout-vent length. NC = neck circumference. BTC = base of tail circumference. HL = head length. HW = head width and HD = head depth

4.3 RESULTS

Results from the regression analysis between body and head variables and snout-vent length (SVL) and the respective regression equations are shown in Figure 20 and Table 7. The linear regression equations had a high coefficient of determination ($r^2 > 0.97$), meaning that there was an apparent lack of morphological variation in all body and head size traits. From these regression equations, TL can be estimated from HL, where TL = 7.43 HL – 10.74.
There were no significant differences between juvenile or sub-adults males and females ($p > 0.05$). Sexual dimorphism was observed in adults with males reaching a larger size than females ($p = 0.01$, Table 8). In addition, adult females had more elongated heads than males (Table 8 and Figure 21).

Examination of the changes in morphometric ratios as a function of increasing body size allows an understanding of growth in *C. niloticus* (Figure 22). There were no significant differences in growth patterns between males and females. The tail and head were relatively long in hatchlings and juveniles compared to body length, though the allometry of the tail was only slight once animals reached 70 cm SVL (Figure 22b and e). The trunk grew at a proportionally faster rate than the head (Figure 22c). There was a pronounced allometry between HL and SVL (Figure 22b). Head width grew slower in animals smaller than 30 cm SVL, but then grew proportionally to SVL (Figure 22d). The same pattern was observed in HD (Figure 22f). Head length increased at a proportionally greater rate than HW and HD in crocodiles smaller than 40 cm SVL, but thereafter HW increased proportionally faster than HL (Figure 22g). Head depth grew proportionally with HL until crocodiles reached 120 cm SVL and then HD increased proportionally faster (Figure 22h

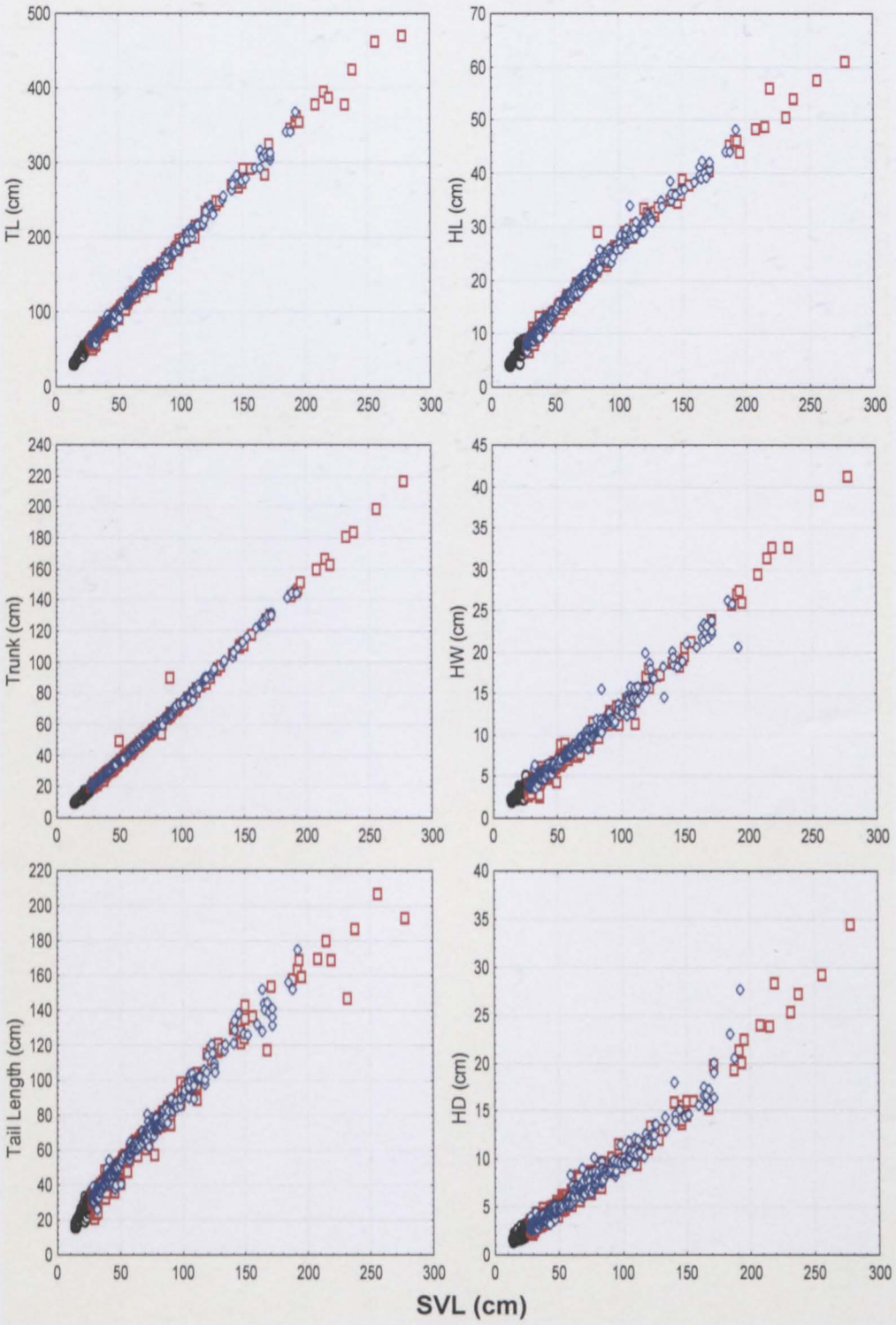

Figure 20. Allometry of body and head morphometrics against SVL (snout-vent length). Regression equations are shown in Table 1. TL = total length. HL = head length. HW = head width. HD = head depth. Red squares = males, blue circles = females and black circles = unknown gender.

Table 7. Linear regression equations (y = ax + b) for body and head morphometrics against SVL.

Y	X	a	b	p	r^2	N
Allometries of body dimensions *vs* SVL						
TL	SVL	1.8056	9.2158	0.000	0.9967	1447
Trunk	SVL	0.7577	-1.4589	0.000	0.9981	1447
Tail length	SVL	0.8056	9.2158	0.000	0.9835	1447
Allometries of head dimensions *vs* SVL						
HL	SVL	0.2431	1.447	0.000	0.9894	1447
HW	SVL	0.1328	0.0892	0.000	0.9869	1447
HD	SVL	0.105	-0.1855	0.000	0.9757	1447

Table 8. Sexual dimorphism of body size and shape in adult *C. niloticus*. The last three columns show results from unpaired two-tailed t-tests for sexual size dimorphism. Statistical tests were performed on log transformed data. Measurements are in cm (± 1.0 mm).

	Female		Male				
Trait	N	Mean ± SD	N	Mean ± SD	t-value	df	p
TL	26	285.47 ± 36.92	24	328.89 ± 67.90	-2.84	48.00	**0.01**
SVL	26	153.87 ± 21.07	24	179.99 ± 42.73	-2.77	48.00	**0.01**
BTC	26	73.73 ± 12.64	24	85.55 ± 24.42	-2.17	48.00	**0.03**
NC	26	75.62 ± 12.40	24	88.47 ± 26.77	-2.21	48.00	**0.03**
HL	26	37.89 ± 4.50	24	42.87 ± 8.44	-2.63	48.00	**0.01**
HW	25	20.49 ± 2.80	22	24.66 ± 7.20	-2.68	45.00	**0.01**
HD	26	16.25 ± 3.55	24	19.53 ± 6.17	-2.33	48.00	**0.02**
tail L	26	131.60 ± 16.79	24	148.90 ± 26.64	-2.77	48.00	**0.01**
TL/SVL	26	1.86 ± 0.05	24	1.84 ± 0.08	0.95	48.00	0.35
BTC/SVL	26	0.48 ± 0.03	24	0.47 ± 0.03	0.68	48.00	0.50
NC/SVL	26	0.49 ± 0.03	24	0.49 ± 0.04	0.41	48.00	0.68
HL/SVL	26	0.25 ± 0.01	24	0.24 ± 0.01	2.12	48.00	**0.04**
HW/SVL	25	0.13 ± 0.01	22	0.14 ± 0.01	-1.41	45.00	0.17
HD/SVL	26	0.11 ± 0.01	24	0.11 ± 0.01	-0.59	48.00	0.56
Tail/SVL	26	0.86 ± 0.05	24	0.84 ± 0.08	0.95	48.00	0.35
HW/HL	25	0.55 ± 0.02	22	0.57 ± 0.05	-2.23	45.00	**0.03**
HD/HL	26	0.43 ± 0.05	24	0.45 ± 0.06	-1.44	48.00	0.16

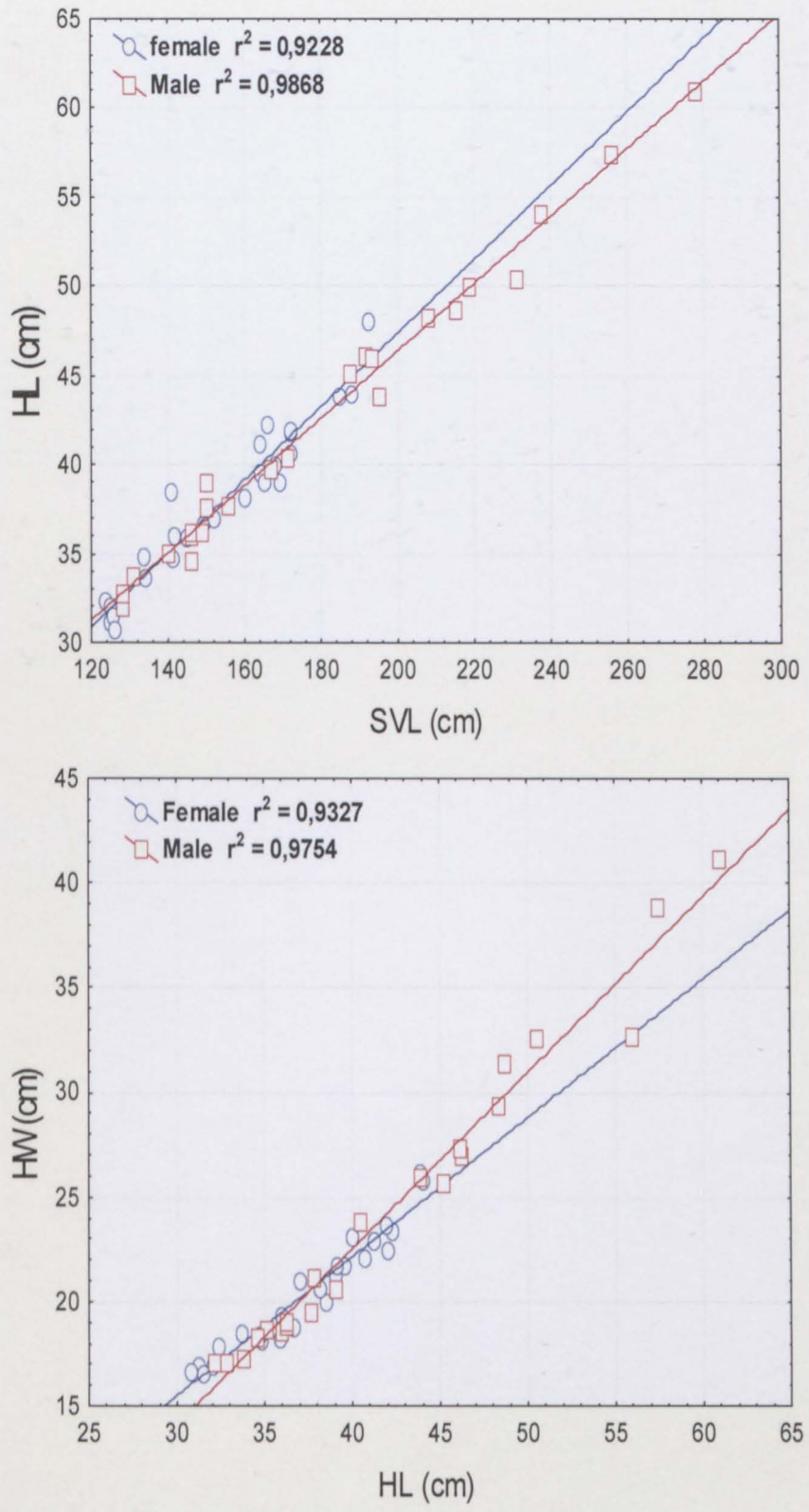

Figure 21. Sexual dimorphism in head length and head shape in adult C. *niloticus*. Circles = females and squares = males.

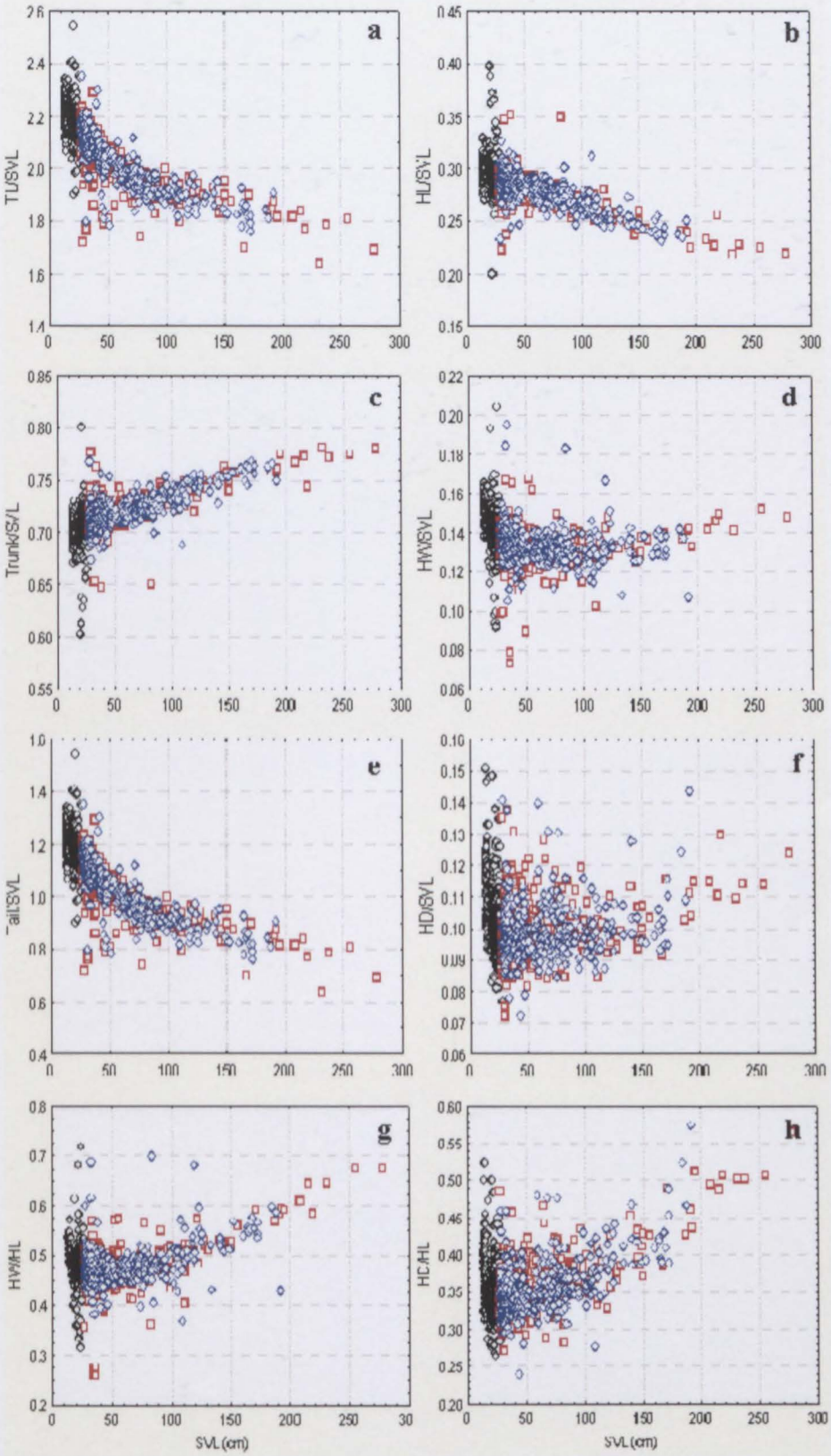

Figure 22. Graphs demonstrating morphometric ratios as functions of snout-vent length (SVL). TL = total length. HL = head length. HW = head width and HD = head depth. Red squares = males, blue circles = females and black circles = unknown gender.

4.4 DISCUSSION

The coefficients of determination (r^2) concerning both head size and body size variables were extremely high. The main biological meaning is the apparent lack of morphological variation on the pattern studied (Verdade, 2000). The regression equations can be useful for predicting body length from head size variables, especially for the study of museum collections or even poaching wastes, in which only crania are usually found intact. In addition, the regression equation of HL to SVL were very similar to *C. niloticus* in South Africa (Leslie, 1997) and may be used when estimating size class of crocodiles during spotlight surveys, when only the head of the animal is above water by using the following equation:

TL = 7.43 HL – 10.74

As in other crocodilian species, males attained a much larger body size than females. Females > 350 cm in TL are rare, whereas males > 450 cm are not uncommon. The reason for this difference is due to the enormous drain on body reserves that the female undergoes during reproduction. Only 60 % of female *C. niloticus* in the Okavango Delta reproduce every year (Chapter 2), which means that most females lack the body reserves to produce a clutch of eggs each year as observed in other wild crocodilian populations (Lance, 1989; Kofron, 1990; Guillette *et al.* 1997).

With regards to body morphometrics, previous studies on *C. porosus* observed significant differences between sexes relative to tail length (Webb & Messel, 1978), where adult males had longer tails than adult females. This could indicate that males of this species are more mobile than females, as demonstrated in the alligator, *Alligator mississippiensis* (Joanen & McNease, 1970; 1972). In this study, no SSD in any body traits was observed in any given size class.

Sexual size dimorphism was demonstrated with regards to head morphology, where males experienced a more pronounced broadening of the head once reaching sexual maturity and adult female heads were more elongated. The broadening of the head occurred in all specimens over 60 cm SVL coinciding with a shift in the crocodiles diet from small mammals to fish (Wallace, 2006) which allows for larger prey capture and a relative gain in energy and growth (Webb *et al.*, 1978). The difference in skull morphology in adults could be used as a

visual recognition of gender (Verdade, 2000) and could also play a role in male-male competition for access to females.

The results from this study demonstrated that SSD only occurred in adults. Sexual size dimorphism might still exist in the smaller size classes but could be masked by different growth rates between males and females, which can be observed in captive reared animals (Jacobsen & Kushlan, 1989). Wild growth rates of *C. niloticus* in the Okavango system are not yet known, therefore, we were unable to conclude if SSD occurred before animals reach sexual maturity.

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CHAPTER 5
SUMMARY AND RECOMMENDATIONS

5.1 SUMMARY

The general summary of the reproductive events during the natural reproductive cycle of *Crocodylus niloticus* in the Okavango Delta, Botswana, is shown in Figure 23.

The general pattern and timing of the different events are very similar to that of *C. niloticus* in the Zimbabwe (Kofron, 1990). This is understandable as both countries share similar climatic conditions. One of the primary differences noted between the two crocodile populations is the size at which crocodiles reach sexual maturity, which seems to be at a smaller TL in the Okavango system.

Okavango female crocodiles reach sexual maturity at around 230 cm in total length (TL) while sexual maturity in females is attained at 262 cm TL in Zimbabwe. However, one should take the different methods applied to determine size at sexual maturity, into consideration. In this study, size at sexual maturity was determined by identifying females that produced vitellogenin. They were therefore developing ovarian follicles, whereas in Kofron's study, abdomen palpation was used in order to detect the presence of large ovarian follicles or oviductal eggs, which is not a very reliable method.

The size at which male *C. niloticus* reach sexual maturity in the Okavango Delta system is still unclear. Only 11 sperm samples were collected between June and October when spermatozoa were present in the semen (Figure 23). One of the samples was from a male just under 200 cm TL, whereas the other 10 samples were collected from crocodiles over 270 cm TL. More data is required, especially from animals between 200 cm and 270 cm TL in order to determine with certainty at what size males attain sexual maturity.

In addition, research is needed regarding growth rates in various wild *C. niloticus* populations, as it might differ from one population to another and will affect the size at which crocodiles reach sexual maturity. This could possibly explain the different findings between the various studies on this species (Cott, 1961; Graham, 1968; Kofron, 1990). Once wild growth rates and

size at sexual maturity are fully understood, we may be able to better comprehend sexual size dimorphism (SSD) in *C. niloticus* and therefore the proximate and ultimate causes of SSD in general (Shine, 1989; Anderson and Vitt, 1990; Stamps, 1993; Andrew and Stamps, 1994; Watkins, 1996; Fairbairn, 1997; Beaupre *et al.*, 1998).

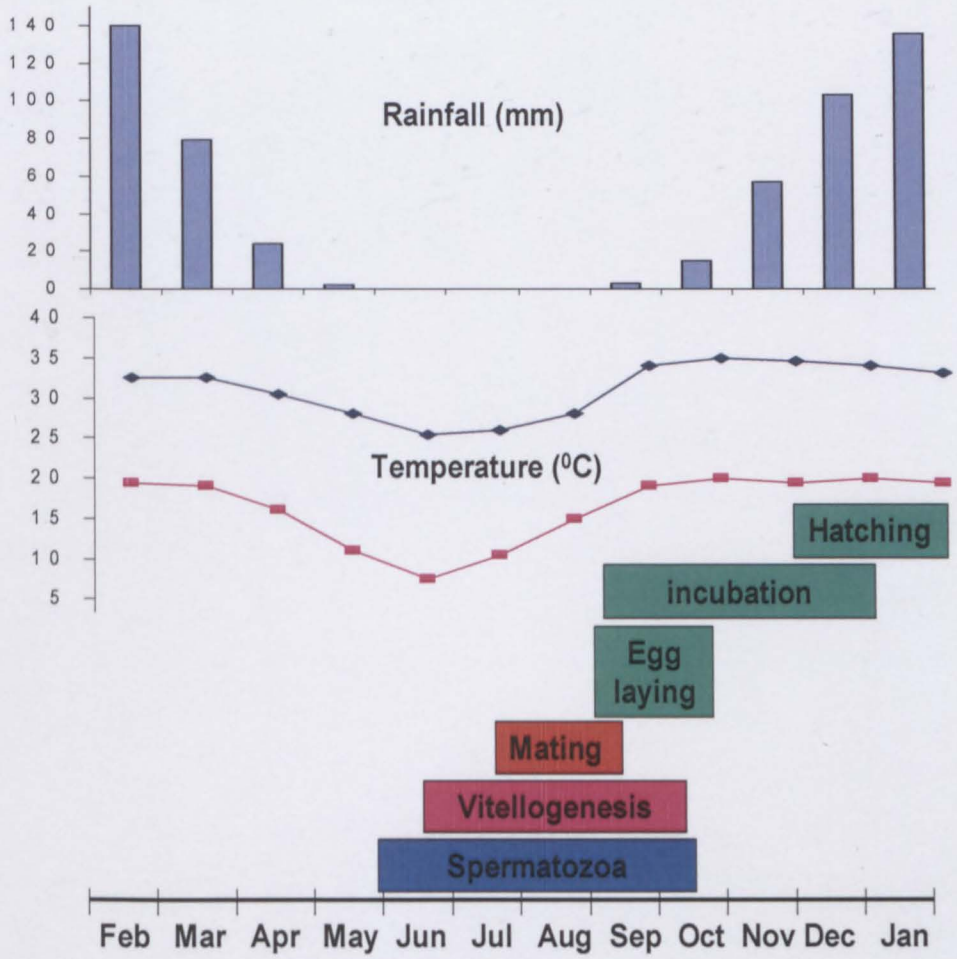

Figure 23. A summary of the reproductive cycle of male and female *Crocodylus niloticus* in the Okavango Delta, Botswana.

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