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Plasma Testosterone and Seasonal Reproductive Changes in the Scorpion Mud Turtle

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Abstract.- The scorpion mud turtle (*Kinosternon scorpioides*) is a freshwater chelonian that is consumed by local populations in states of Pará and Maranhão, Brazil. Current analysis evaluates the reproductive parameters of recently captured turtles. Turtles underwent histological analysis of testes and hormone levels to establish and investigate the species´s natural cycle in different stages, with special focus on aestivation behavior as it relates to such as reproductive strategies. This information will help inform sustainable strategies of conservation and reproduction which would contribute towards studies on other animals with similar behaviors. Twenty adult male animals were dissected and their testes removed for the morphometric analysis of epithelial thickness and tubular and luminal diameters. Testosterone concentrations were measured by radioimmunoassay. Results indicated that the hormonal testosterone profile was linked to the mating season and the highest spermatogenic activity occurred during the rainy season when testosterone concentrations were lower than those during the dry season. Biological observations on the reproductive behavior revealed that seasonal variations in gonad size, morphological characteristics of the testes and hormone concentrations all indicate that the environmental conditions of the scorpion mud turtle´s natural habitat influenced reproductive seasonality.

Key words: Hormone, *Kinosternon scorpioides*, seasonality; testis morphometry, wild fauna.

INTRODUCTION

The scorpion mud turtle (*Kinosternon scorpioides*) is a freshwater turtle of the family Kinosternidae, which has both aquatic and terrestrial habits and feeding on fish, tadpoles, adult amphibians, insects, algae, plant debris, crustaceans and gastropods (Vanzolini *et al*., 1980; Acuña-Mesen, 1994). The scorpion mud turtle is widely distributed across Central and South America, from sea level up to an elevation of 2,500 m (Acuña-Mesen, 1994). It is present in the North and northeastern regions of Brazil (Trebbau and Pritchard, 1984) and may be found in fields and along river banks of the Maranhão lowlands (Vanzolini *et al*., 1980), with a large populations known in the vicinity of the town of São Bento, MA, Brazil (Viana *et al*., 2014a).

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The state of Maranhão is a region featuring distinct rainy and a dry seasons: there are extensive flooded areas and swamps from January to June when fishing becomes the main subsistence activity for humans; and, at the end of this season and the beginning of summer, between July and December, these areas become dry and crops are cultivated on a subsistence basis. Although the turtle is an important source of protein and has an economic value to the local populations of Maranhão, little is known about its reproduction in the wild (Pereira *et al*., 2007). The turtles begin their aestivation processes during the dry season when they bury themselves in the soil. This behavior reduces their basal metabolism during the season when the environment is not favorable for activity. It has been reported that the reproductive cycle of several animals depends on hormonal control that directly produces the physiological changes of the reproductive system during the mating season (Reddy and Prasad, 1970). Testosterone affects the size of the testes when compared to body size, and stimulates typical sound changes of mating behavior

and territorial defense (Licht *et al*., 1985a).

Studies on gonad cycles of freshwater chelonians have been based on histological investigations (Moll, 1979; Licht, 1984) and on the seasonal changes of steroid levels (Licht, 1982; Licht *et al*., 1980, 1985a,b; Mahmoud *et al*., 1985; Mendonça and Licht, 1986) in lizards *Phymaturus punae* (Boretto *et al*., 2014). Studies from our scientific team have contributed towards a deeper knowledge of *K. scorpioides* in different climatic seasons, such as researches related to venous plexus description (Pereira *et al*., 2011), embryologic development (Anunciação *et al*., 2011, 2012), epididymal (Viana *et al.*, 2013) and vas deferens morphology (Viana *et al.*, 2014b), and morphological aspects of female from capitivity (Chaves *et al.*, 2012) . Sousa *et al.* (2014) based on the acrosome development in spermatids and the overall germ cell associations have revealed that ten stages of the seminiferous epithelium cycle were characterized in *K. scorpioides*.

Our work analyzes the reproductive characteristics of newly captured animals through histological analysis and the evaluation of testosterone concentrations so that the species natural cycle at different stages of the year may be established and interpreted. The assay may also be a contribution towards developing sustainable strategies of conservation and reproduction, with special focus on the aestivation behavior as a reproduction strategy for the conservation of the species.

MATERIALS AND METHODS

Animals and laboratory

Adult male scorpion mud turtles (n = 20) were captured alive in the town of São Bento, in the lowlands of the state of Maranhão, Brazil, Quality control parameters of hormonal assays were analyzed according to routine procedures applied at the Hormonal Dosage Laboratory, Department of Reproduction, School of Veterinary Medicine and Animal Science of the University of São Paulo.

Collection and euthanasia

Animals were captured regularly at 3-month intervals, between December 2010 and September 2011. Two collections were done at each season, or rather, at the peak of the rainy season (March) and during the transition to the dry season (June); similarly at the beginning of the dry season (September), and during the transition to the rainy season (December). Temperature, humidity and rainfall were recorded accordingly at each collection (Table I). All twenty animals were anesthetized with xylazine 2% (40mg/kg/IM) and ketamine hydrochloride 1% (60mg/kg/IM) and euthanized with thiopental sodium 2.5% (60mg/kg/EV) by catheterization of the cervical venous sinus, according to technique by Schumacher (1996). The coelomic cavity was subsequently opened with a steel handsaw to disarticulate the bone bridge that joins the carapace to the plastron. The gonads were removed and the testes isolated for subsequent microscopy.

Table I.- Mean and standard deviation rates of temperature, humidity and rainfall in the Baixada Maranhense MA Brazil, according to the season.

Period of the year	Temperature (ºC)	Humidity (%)	Rainfall (mm)
December/2010	27.52±1.23a	75.97±8.30a	6.57±16.58a
March/2011	26.32±0.70b	85.93±4.39b	2.37±4.31b
June/2011	26.51±0.62 b	80.55±3.36c	0.79±2.47b
September/2011	27.58±0.49 a	71.70±3.05a	0.01±0.07b

Means with different letters in the same row indicate statistical difference ($p<0.05$) for Student-Newman-Keuls (SNK) test; test of normality Cramer-von Mises W-Sq 0.03979, Pr>W-Sq>0.2500.

Histological studies

Testes were fixed in 4% buffered formaldehyde for 12 h, and then processed by routine techniques for paraffin embedding and 5-µm thick histological sections were cut which were stained with hematoxylin-eosin and Masson trichrome. Images for morphometric studies were obtained with a binocular microscope (Olympus BH-41, São Paulo, Brazil) equipped with a digital camera.

Morphometry of seminiferous tubules

Images for morphometric studies were obtained with a binocular microscope (Olympus

BH-41, São Paulo, Brazil), equipped with digital camera for photographic record. Histomorphometric analyses were performed with program (GIMP 2, Klaus Goelker, Cambridge, UK) to measure the average rates of epithelial height and tubular and lumen diameters of the seminiferous tubules. Rates were obtained by the micrometer eyepiece adapted to the microscope. In the case of the tubular sections, ten slides with three serial sections were made. Tubules were lined at the base of the epithelium (at the concentration of the basal membrane) to obtain total tubular diameter, and near the apical edge to obtain luminal diameter. A 10x objective lens was employed to measure the epididymis and vas deferens and a 20x objective lens to measure the seminiferous tubules, whereas a 40x objective lens was used to measure the height of the seminiferous epithelium.

Scanning electron microscopy (SEM)

Pieces of testes were fixed in 2.5% gluteraldehyde then froze for 72 h. They were then cryofracturated in liquid nitrogen, washed in 0.1 M phosphate buffer, postfixed with 1% osmium tetroxide and dehydrated in a series of increasing alcohol rates (50 to 100%). Samples were dried with a critical-point apparatus Balzers CPD 020, Balzers Union Ltd, Liechtenstein) with liquid CO_2, and mounted on aluminum stubs by carbon paste. The samples were then sputter-coated with gold (Emitech K550, Emitech Ltd. Ashford, Kent, UK), analyzed and photographed under a scanning electron microscope (LEO 435VP, Zeiss, Cambridge, UK).

Transmission electron microscopy (TEM)

Pieces of testes were fixed in 2.5% glutaraldehyde, washed in 0.1 M phosphate buffer and postfixed in 1% osmium tetroxide. They were subsequently dehydrated in a series of increasing alcohol rates (50 to 100 %) in propylene oxide and resin. The mixture was replaced by pure resin and poured into molds. Ultra-thin sections were collected on copper screens and contrasted with 2% uranyl acetate solution and 5% lead citrate solution. Samples were analyzed with a transmission electron microscope (Morgagni 268d, FEI Company, Tokyo, Japan).

Testosterone concentrations

One ml blood samples from each animal was collected in the rainy and dry seasons. Blood, collected directly with syringe and 25 x 7 needles from the dorsal cervical sinus, centrifuged and the serum stored in 1.5 ml microcentrifuge tubes and frozen at -20ºC (Owens, 1980). Serum testosterone was measured by radioimmunoassay (RIA) technique in solid phase with the commercial diagnostic set (Coatá-Count Testosterone-Diagnostic Products Corporation, Los Angeles, CA, USA) developed for quantitative measurement of testosterone in human serum. These diagnostic sets use ^{125}I-labeled hormone as tracer and show little cross-reaction with precursors specific for each hormone studied (Shah *et al*., 1995).

Statistical analysis

The analysis of variance was performed with GraphPad InSat program to obtain mean and standard deviation; Cramer-von Mises test for normality checked homoscedasticity between the variables; Student-Newman-Keuls (SNK) comparison average test provided morphometric rates. Hormonal and biometrics variables were unstable with coefficient of variation between $15\% < CV < 30\%$, at significance concentration $p < 0.05$ (Viana *et al*., 2012).

RESULTS

Mass and measurement of testes

Depending on the season, the right and left testes had higher average length and width. Difference in the length of the left testis was observed in September when compared to that in other months (Table II), although there was no significant difference in testis mass between the seasons (Table III).

Histological structure of testes

The testes of *K. scorpioides*, covered by the tunica albuginea, were composed of dense connective tissues with collagen fibers and with branching blood vessels mediated by lymphatic spaces (Figs. 1A and 1B). The seminiferous tubules had irregular arrangements in the interstitium (Fig. 1D), while the basal compartment was surrounded

Table II.- Seasonal comparisons of the means and standard deviations of biometric parameters (cm) of the testes of *K. scorpioides*.

Measures	Rainy season		Dry season	
	Mar/11	Jun/11	Sept/11	Dec/10
RT - L	1.43 ± 0.40a	1.36 ± 0.36a	1.30 ± 0.76a	1.31 ± 0.32a
RT - W	1.43 ± 0.26a	1.11 ± 0.27a	0.77 ± 0.15a	1.13 ± 0.26a
LT - L	0.70 ± 0.30a	1.30 ± 0.37a	1.24 ± 0.41b	1,31 ± 0,37a
LT - W	0.52 ± 0.41a	1.22 ± 0.24a	1.33 ± 0.59a	1.13 ± 0.21a

RT-L, length of right testicle; RT-W, width of right testicle; LT–L, length of left testicle; LT-W, width of left testicle. Means with different letters in the same row indicate statistical difference ($p<0.05$) for Student-Newman-Keuls (SNK) test; test of normality Cramer-von Mises W-Sq 0.03979, Pr>W-Sq>0.2500.

Table III.- Seasonal comparisons of the means and standard deviations of average mass (g) of the testes of *K. scorpioides*.

Measures	Rainy season		Dry season	
	Mar	Jun	Sep	Dec
RT	0.18 ± 0.07a	0.31 ± 0.14a	0.25 ± 0.06a	0.34 ± 0.12a
LT	0.52 ± 0.41a	0.23 ± 0.09a	0.23 ± 0.06a	0.23 ± 0.08a

For abbreviations and other detail see Table II.

by the basal membrane and contained spermatogonia, spermatocytes (at the beginning of the stratification of the seminiferous epithelium) and Sertoli cells with irregular nucleus whose function is to nurture the germ cells for sperm formation. Spermatocytes, round and elongated spermatids, and spermatozoids were observed in the adluminal compartment. The seminiferous epithelial cycle of germ cells, such as spermatogonia, spermatocytes, and spermatids, was observed. Whereas spermatogonia were ovoid-shaped immature cells in the basal compartment and primary or secondary spermatocytes were the biggest cells, spermatids were rounded or elongated with smaller nucleus and less cytoplasm. Comma-shaped spermatozoids were found in the tubular lumen (Figs. 1C, 1D). The seminiferous tubules showed morphological changes according to seasons. Although in the rainy season (January to June), the epithelium was intact (Figs. 2A, 2B), during the dry season (July to December), spermatogenic activity was not observed in the seminiferous tubules and the epithelium was disorganized (Figs. 2C, 2D).

Ultrastructure of testes

SEM studies showed spermatogonia-like and spermatozoa-like cells in the seminiferous epithelium during the rainy season. The seminiferous epithelium had only spermatogonia-like cells during the dry season (Fig. 3). When observed under the TEM, the seminiferous epithelium of the testes contained spermatogonia, spermatocytes, spermatids and spermatozoa in the rainy season. During the dry season, only spermatogonia, spermatocytes, and Sertoli cells were present (Fig. 4).

Table IV.- Seasonal comparison of the means and standard deviations of morphometry (mm) of tubular and luminal diameters and height of the seminiferous tubules of the *K. scorpioides*.

Seminiferous tubules	Season of the year			
	Rainy season		Dry season	
	Mar/11	Jun/11	Sept/11	Dec/10
Tubular diameter	368.62 ± 45.20 a	291.25 ± 40.50 b	202.37 ± 30.16 c	187.70 ± 25.56 c
Luminal diameter	180.52 ± 40.51 a	144.02 ± 36.00 b	134.31 ± 31.40 c	174.02 ± 22.63 a
Epithelial height	96.,82 ± 16.77 a	66.91 ± 13.16 b	36.68 ± 12.87 c	30.40 ± 6.64 c

For statistical details, see Table I.

Morphometry of seminiferous tubules

The morphometry of the tubular and luminal diameters and thickness of the seminiferous tubules were significantly different ($p<0.05$) between the seasons (Table IV). During the rainy season, rates varied significantly ($p<0.05$), whereas in the dry season the average tubular and luminal diameters and thickness decreased. The above confirms that active sperma- togenesis occurs during the rainy season.

Hormone dosage for testosterone

Testosterone concentration averaged 142.79±141.35 ng/dL in December or late drought

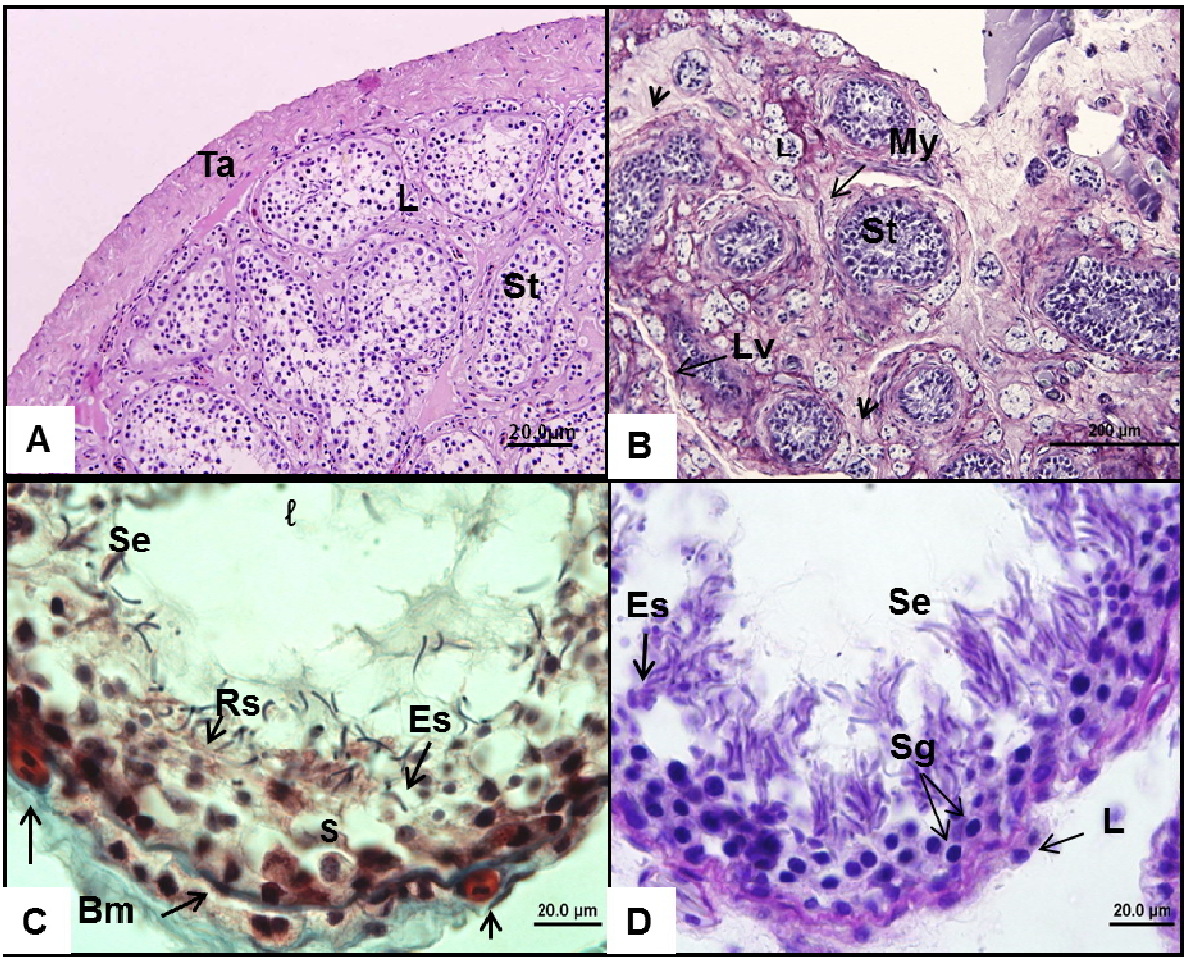

Fig. 1. Cross-sections of the testes of the *K. scorpioides*. A and B: tunica albuginea (Ta), seminiferous tubules (St), Leydig cell (L), myoid cells (My) and lymphatic vessels (Lv). C and D: Tubular lumen (ℓ), seminiferous tubule delimited by basal membrane (Bm), stratified seminiferous epithelium (Se), blood vessels (Bv) (arrow), Sertoli cell (S) and spermatogonia (Sg), round spermatids (Rs) and elongated (Es) (A, B and D: HE, C: Masson's Trichrome). LM.

Table V.- Comparisons of the means and standard deviations of testosterone concentrations (ng/dL) in the *K. scorpioides*.

Animal	Season of the year			
	Rainy season		Dry season	
	Mar/11	Jun/11	Sept/11	Dec/10
1	1412.10	1288.32	1000.90	300.96
2	10.44	26.45	1425.10	15.45
3	1584.10	1311.2	1403.00	283.90
4	1622.00	646.82	1628.22	12.12
5	1322.60	153.33	1244.87	101.54
x ± s	1190.25 ± 670.85 a	685.22 ± 607.02 ab	1340.42 ± 233.62 a	142.79 ± 141.35 b

For statistical details, see Table I.

season (early winter), while the first testosterone concentration increase (1190.25±670.85ng/dL) occurred in March during the rainy season. In June, transition period from the rainy to the dry season (early summer), testosterone decreased (685.22± 607.02 ng/dL) and a second increase (1340.233± 42.62 ng/dL) occurred in September (Table V).

DISCUSSION

Mean serum concentrations of testosterone during the seasons studied suggest that in the natural environment, distinct seasonality in the reproductive cycle of this species exists, evidenced by the

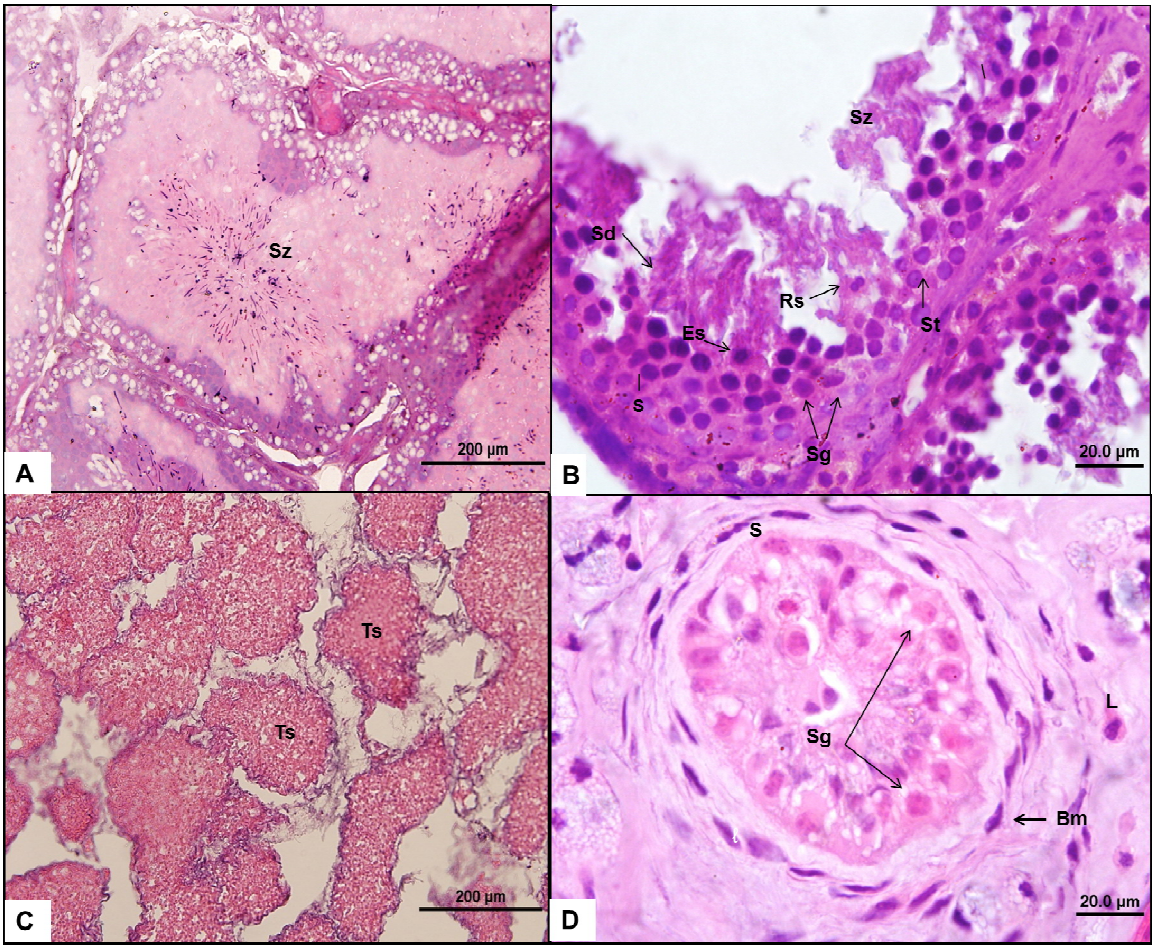

Fig. 2. Testes of the *K. scorpioides* in wet and dry seasons. A and B: Histology of the seminiferous epithelium in the rainy season, with presence of spermatogonia (Sg), spermatocytes (St), round spermatids (Rs) and elongated (Es) and spermatozoa (Sz). C and D: Histology of the seminiferous tubules (Ts) of the dry season without development of the germinal epithelium, and with presence of spermatogonia (Sg), Sertoli cells (S), Leydig cells (L) and basal membrane (Bm). (A, B, C and D: HE). LM.

significant differences in peak rates of testosterone between the wet and dry seasons. The morphological features were similar to showed by specimens from breeding center (Araujo *et al*., 2012).

Lowest rates in hormone concentration occurred in December with little activity in the seminiferous epithelial cycle and, consequently, a concurrent decrease in spermatogenesis. These results coincided with the height of the dry season and its high temperatures when the animals buried themselves in the soil to maintain a low basal metabolism, a behavior known as aestivation, very similar to hibernation (Randall *et al*., 2000).

The results of serum samples obtained during the season of intense rains (March) showed high testosterone increases, coinciding with the time when males maintain territories and choose females. This increase may also be explained by the stress of confrontation for territorial domination. Our histological studies showed that the testes during this specific season contained all types of spermatogenic cells, including mature spermatozoa, and this fact indicated an active spermatogenic process. In addition, spermatocytes and Leydig cells were present in larger numbers than in the dry season. Moreover, the right and left testes were at their highest average mass, lengths and widths, and thus confirm reproductive activity during that season. However, there was no significant

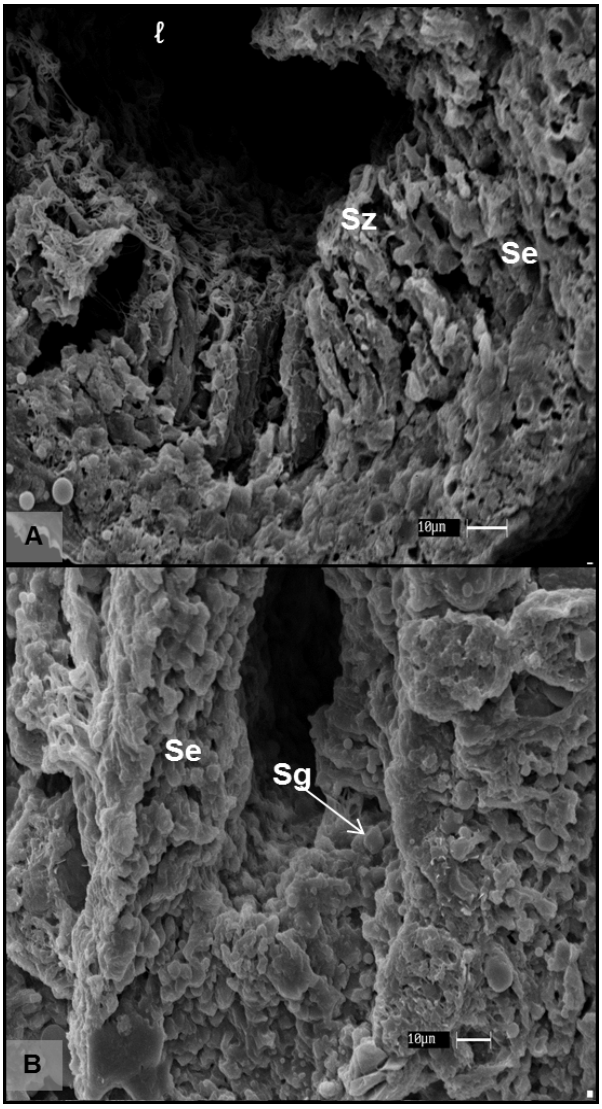

Fig. 3. Testes of the *K. scorpioides* in wet and dry seasons. A: Seminiferous epithelium (Se) in the rainy season, lumen (ℓ) with the presence of spermatozoa-like (Sz). B: seminiferous epithelium (Se) of the dry season with the presence of spermatogonia-like (Sg). Bars: 10 mm and 10 mm. SEM.

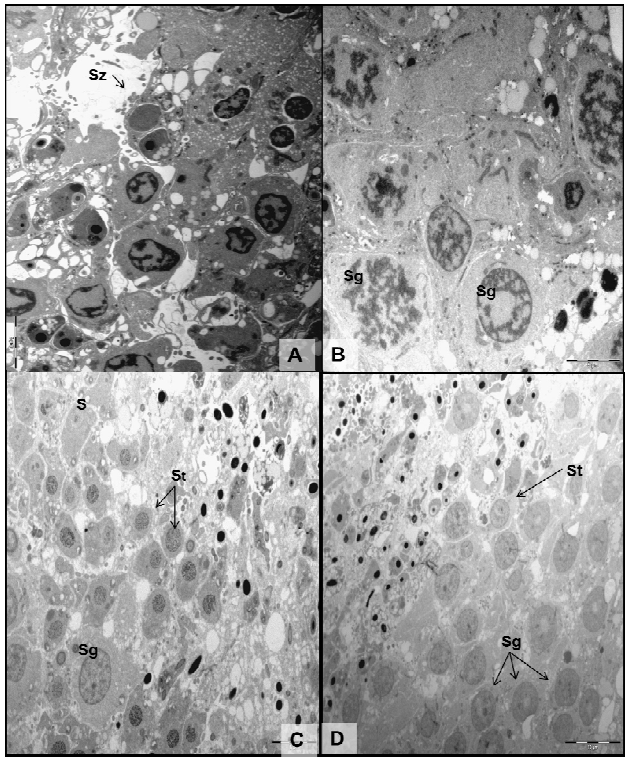

Fig. 4. Testes of the *K. scorpioides* in wet and dry season. A and B: seminiferous epithelium in the rainy season, with the presence of spermatogonia (Sg), spermatocyte (St), spermatid (Sd) and spermatozoa (Sz) (arrow). Bars: 60 µM, 5 µM. C and D: seminiferous epithelium in the dry season, with the presence of spermatogonia (Sg) and spermatocyte (St), Sertoli cells (S). Bars: 50 µm and 10 µm. TEM.

difference in testis mass between the seasons. The above feature has also been observed in quail *Coturnix coturnix*, where no obvious change in testis mass between the reproductive and non-reproductive seasons was reported (Amoroso *et al*., 2008).

During the transition season, from the end of the rainy to the early dry season, represented by the collection in June, there was decrease in testosterone when compared to the peak recorded during the rainy season (March). This may have occurred because males had already marked their territories even though normal spermatogenesis was observed histologically.

It was presumed that mating might have occurred in September during the time of the second testosterone increase. At that time, the lowest standard deviation, when compared to average, was observed by uniform testosterone plasma concentrations. The highest temperatures for the lowlands in Maranhão, recorded during this season, coincided with a decrease in spermatogenic activity, already characterized histologically. The

spermatozoa was stored in the epididymis waiting for the right reproduction moment, as described in turtles *Trachemys scripta* in the state of Ohio, USA (Gribbins *et al*., 2003). The above study reported that mature sperm was stored in the epididymis until reproduction time in the following spring. It was a sure indication that environment and climate influenced the reproductive process. McPherson and Marion (1981) studied *Sternotherus odoratus* from Alabama, and noted in the months of July to September that the epididymis are lighter and the testicles are heavier, so it is possible to state that there is a movement of spermatozoids. When the testicles are full, the epididymis is in a lower density of spermatozoids in *K. scorpioides* (Viana *et al*., 2013). As noted in *Sternotherus odoratus* by McPherson and Marion (1981), the epididymis are full of spermatozoids throughout the year in *K. scorpioides*, although in smaller quantities between periods, but, so far, it is not possible to do statements on viability (Viana *et al*., 2013).

In our study *K. scorpioides* showed hormonal activity according to seasons, similar to other chelonian species, such as the turtles *Lepidochelys kempi* from the British West Indies (Caribbean territory) (Rostal *et al*., 1997) and *Trionyx sinensis* from southern China (Lofts and Tsui, 1977). Testosterone concentrations in these animals were high (844 ng/dL) during the pre-mating season. However, unlike the features observed in the turtles in our study where testosterone increased during the mating season, testosterone concentrations fell during the mating season (65 ng/dL) in the species described above and intense spermatogenic activity occurred during the hottest time of the year (Lofts and Tsui, 1977; Rostal *et al*., 1997). For the turtles *Gopherus flavomarginatus* studied by Trápaga *et al*. (2000), the males emerge after brumation with low testosterone levels, regressed testes, and mature spermatozoa. Testosterone increases along with day length and ambient temperature and courtship occurs with limited frequency. Testosterone increases steadily toward July as the testes recrudesce although surface activity is limited. The onset of the rainy season significantly increases epigean activity, including mating, and spermatogenesis peaks. Testosterone levels decrease as brumation approaches and the testes regress.

In studies with the turtles *Chrysemys picta*, low testosterone concentrations were observed (30 ng/dL) during the autumn-winter period, preceding hibernation (Ernst, 1971; Ganzhorn and Licht, 1983). These turtle species were different from scorpion mud turtles with two testosterone increases, an apparent characteristic of the species, although differences may be related to different climates and locations.

In male lizards, *Sceloporus jarrovi* (Moore and Marler, 1987), testosterone concentrations were low in the winter and high during the mating season when males displayed intense territorial defense. This is actually similar to our findings in *K. scorpiodes*.

Highest spermatogenic activity occurred during the rainy season, when testosterone concentrations were lower than those during drought. Thus, the existence of reproductive seasonality of scorpion mud turtles in their natural habitat has been described in the Baixada Maranhense.

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