Chapter

Steroidogenesis during Dry and Wet Season of Free-Living Male Scorpion Mud Turtle (*Kinosternon scorpioides*)

Diego Carvalho Viana, Amilton Cesar dos Santos, Alana Lislea de Sousa and Antonio Chaves de Assis Neto

Abstract

This study aimed to elucidate seasonal reproductive patterns by associating the immunolocalization of the steroidogenic enzymes responsible for estrogen and androgen synthesis, cytochromes P450 aromatases (P450 arom), 17-hydroxylase/17,20-lyase (P450c17) respectively, as well as the redox partner NADPH cytochrome P450 oxido-reductase (CPR) in the male mud turtle (*Kinosternon scorpioides*). The animals were collected at São Bento, Maranhão, Brazil. Testicles and epididymes from 38 adult animals were characterized by immunohistochemistry (IHC) and western blotting. Data were analyzed in two seasons: dry and wet season. In addition, the gonadosomatic index, morphometry, concentrations of testosterone, corticosterone, and estradiol were analyzed. The changes in biometrics, cell morphometry, and testicular steroidogenesis between the wet and dry seasons support that estrogen produced by the Leydig cells might influence germ cell production during spermatogenesis, and the expression of the enzymes P450 arom and P450c17 in the Leydig cells that play a role in testicular quiescence. We found that the P450arom, P450c17, and CPR are present in the testes and epididymides at both seasons. These findings support a state of male reproductive quiescence during the dry season and active spermatogenesis in the wet season.

Keywords: aromatase, reproductive seasonality, wild animals, immunohistochemistry, theriogenology

1. Introduction

The scorpion mud turtle (*Kinosternon scorpioides*) is a freshwater turtle distributed geographically in South and Central America [1, 2]. In natural environments, investigations of the biology of the species are essential to ensure sustainability, preservation and the establishment of captive reproductive management plans. One of the reasons would be the vulnerability to predatory hunting during the dry periods of the year, associated to seasonality. Due to the estivation periods, the animals stop reproducing and can still be threatened by anthropic actions.

Investigations into the biological knowledge of species belonging to the Cryptodira suborder, such as the scorpion mud turtle, in natural environments are essential to ensure sustainability, preservation, and the establishment of reproductive management plans, as all species of the Amphichelydia suborder are extinct. Consequently, it is known that the risk of vulnerability is imminent [3], given that these animals have seasonal habits and there is still no specific measurement of the nesting rate [4, 5]. Data on the distribution of *K. scorpioides* in Latin and Central America, revealing its presence across all states of the Amazon region and in nearly all Brazilian continental biomes [6].

The steroidogenic enzymes are responsible for synthesis of sex hormones [7, 8]. In addition, the enzymes immunolocalization change during the reproductive phases. In mammals, the P450arom and P450c17, associated with the reducing enzyme cellobiose dehydrogenase (CDH), are responsible for androgen synthesis from other androgens and from progesterone, respectively [9, 10]. In turtles, aromatase enzyme activity was described in the species *Trachemys scripta*, where P450arom was expressed in the Sertoli and Leydig cells. The study linked the enzyme P450arom with estrogen synthesis, which is important to spermatogenesis and fertility regulation in the maintenance of sertoli cells [11].

Previous studies have examined the reproductive biology of freshwater turtle carried out kept in captivity [12–16]. Therefore, one of our objectives was to understand the behavior of male turtles in the dry and wet seasons, because the females are reproductively active all year round. In The state of Maranhão, Brazil has a wet and dry season: there are extensive wetlands and marshes from January to June, when fishing is the main subsistence activity and between July and December, these areas become dry and subsistence crops are grown [17].

Few studies have dealt with the reproductive cycle of scorpion mud turtle from the aspect of endocrine characteristics [15, 16]. The study aimed at understanding the seasonal reproductive pattern of the male, covering steroidogenic enzymes, their immunolocalization, gonadal hormone levels. The study may to 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.

2. Materials and methods

2.1 Morphology

The animals presented the following anatomic components: one pair of testes, one pair of epididymides and vas deferentes, and a penis (**Figure 1**). Gonadal hormone activity and increases in rainfall coincided seasonally according to average/mean monthly precipitation between November 2010 and October 2014 the data were provided by NuGeo — Geoenvironmental Nucleus of the State University of Maranhão (UEMA), which correlated with events in the gonadal hormone cycle defined according to histological testicular analyses (**Figure 2**). As the data for temperature and light period were constant throughout the seasons studied, attention was paid to rainfall data, which were correlated with reproductive events (**Figure 3**).

2.2 Animals and experimental design

Thirty-eight adult animals were captured on the Northern of Maranhão State, Brazil (2°41′ S, 44°49′ W). The collections were authorized by the ICMBio/MMA

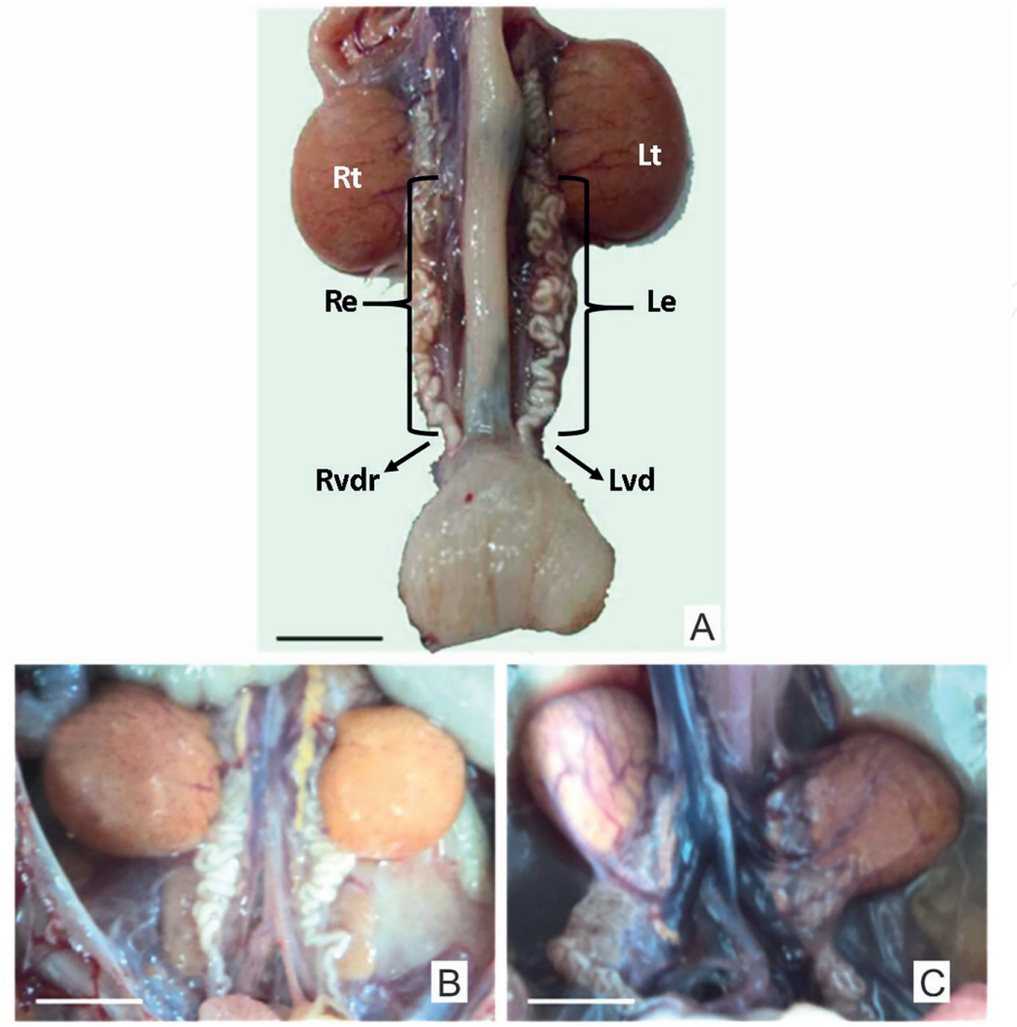

Figure 1.
Anatomy of the male genital apparatus of the jurará turtle (K. scorpioides).: Topographic view (a): Syntopy of the right and left testis (Rt and Lt), right and left epididymis (Re and Le) and right and left vas deferens (Rvdr and Lvd). B: Testis and epididymis, wet season (January–June). C: Testis and epididymis, dry season (July–December). Bar 2 cm.

for activities for scientific purposes (license No. 33021–4), and approved by the Commission of Ethics in the Use of Animals, School of Veterinary Medicine and Animal Science, University of São Paulo, Brazil (protocol 2588/2012).

The animals colleted has a carapace length greather than 10 cm, which were divided into two experimental group: from dry season (n = 18 specimens) - September (n = 5); October (n = 8); December (n = 5), and wet season (n = 20 specimens) March (n = 15), June (n = 5). The combined testis mass (right and left) and body mass were determined using digital electronic scales to obtain the gonadosomatic index (GSI). The weight of the two testes provided the GSI which refers to the percentage of body weight allocated within the gonads. Data were analyzed for mean, standard deviation and coefficient of variation. The equation, $Y = a + bX$, defined the correlation between the width and length and total weight of carapace variables.

2.3 Testis, epididymis, and blood sample collection

The animals were anesthetized with 2% intramuscular (IM) xylazine hydrochloride (40 mg/kg) and 1% ketamine hydrochloride (60 mg/kg/IM), and 2.5% sodium

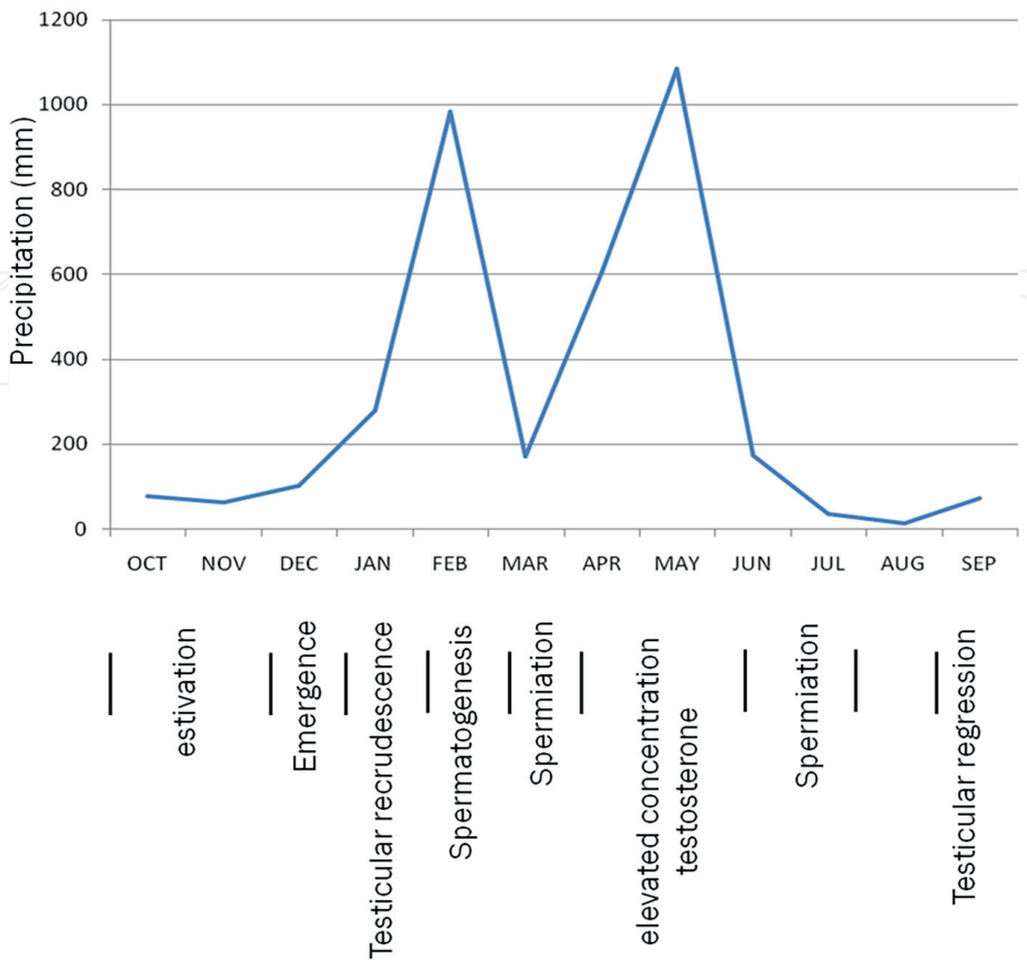

Figure 2.
Mean daily precipitation between November 2010 and October 2014 correlated with important events in the gonadal hormone cycle. Events were defined according to histological testicular analyses.

thiopental (60 mg/Kg/IV) the cavernous sinus [18]. The standard collection time was established at 10:00 h, the animals were captured by hand, and the blood was collected within 4 min. Samples of 3 ml blood were collected from each animal in the wet and dry seasons. For this, the dorsal cavernous sinus was sterilized with iodine and the blood was collected with a 3-ml syringe and a 25 X 7-gauge needle, placed in a tube without heparin and kept on ice for up to 2 h before centrifuging for 15 min at 2000–3000g. Shortly after centrifuging, the serum was transferred to 1.5 ml tubes and frozen at −20°C [19]. A portion of the testicles and epididymis, after collection, were preserved at a temperature of −80°C using liquid nitrogen for molecular analysis.

Later the coelomic cavity was opened using a steel handsaw to disarticulate the bony bridge that joins the carapace to the plastron to remove the viscera and visualize and remove the reproductive tract. Samples of testis and epididymis were sent for processing according to methodology defined. The testicles were weighed on a precision digital balance and measured with a caliper for length and width. Measurements are given in two decimal points. The volume was obtained via a measuring cylinder by checking the volume of water after the addition of the organ.

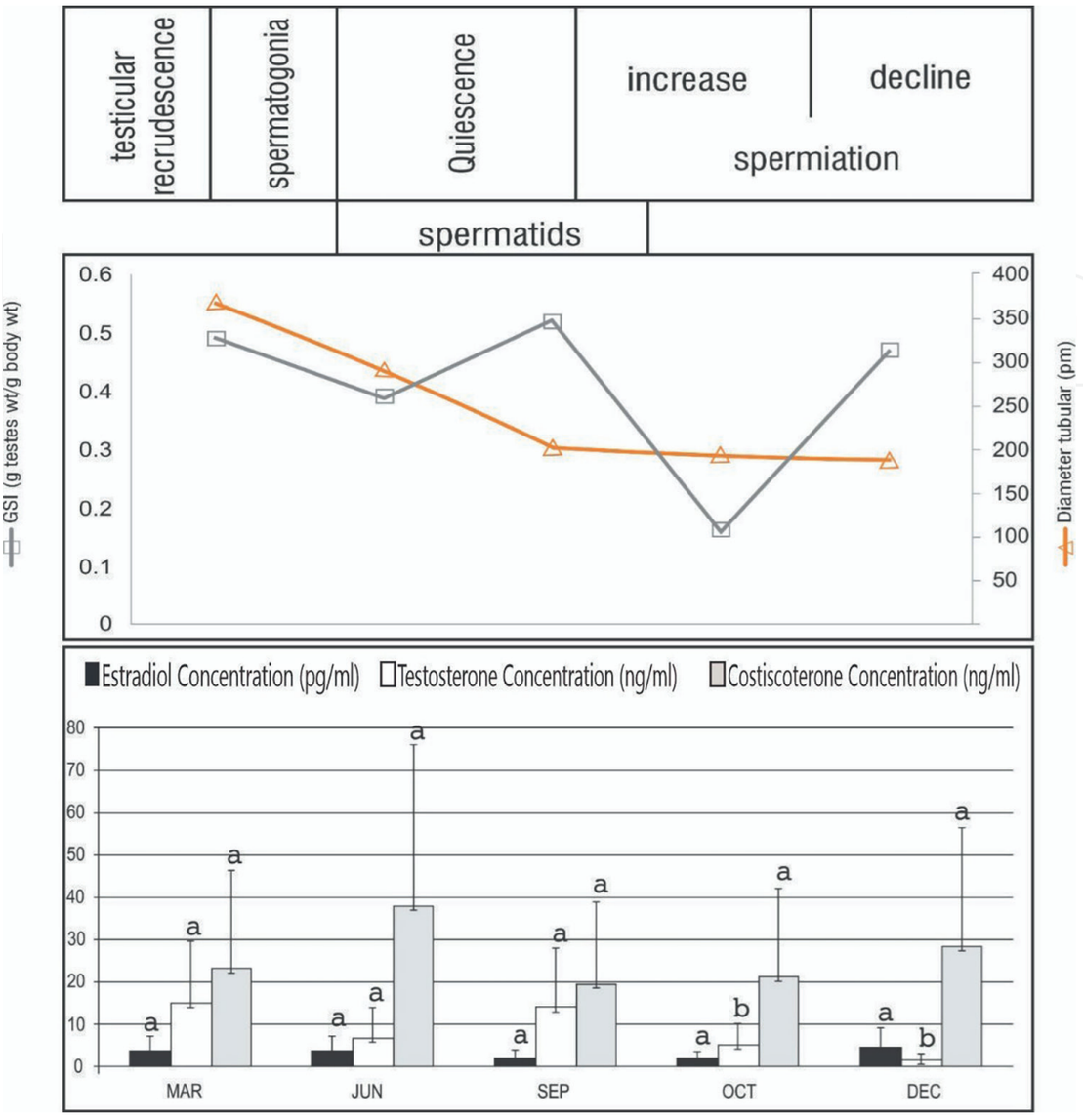

Figure 3.
Mean concentrations of estradiol, costicosterone and testosterone related to the gonad somatic index (IGS), tube diameter and events in the gonadal hormone cycle [wet season - mar (n = 15), Jun (n = 5); dry season - Sep (n = 5); Oct (n = 8); Dec (n = 5)]. Bars with different letters indicate significant differences between the groups (P < 0.05). Mar (march), Jun (June), Sep (September), Oct (October), Dec (December).

2.4 Radioimmunoassay

The concentrations of testosterone, corticosterone, and estradiol were measured by radioimmunoassays using 125I–testosterone, 125I–costicosterone and 125I–17β–estradiol (IM1119 kit for testosterone; SAS Immunotech, Marseille, France and RIA kit 07–120,102 for corticosterone; MP Biomedicals, Irvine, CA, USA; for estradiol CT RIA Kit 07–238,102, MP Biomedicals), respectively (see Viana et al., 2014a for more details). The sensitivity of the test for testosterone (TTT) was 0.1 ng/ml; for corticosterone, 7.7 ng/ml; the interassay and intraassay coefficients of variation for testosterone and corticosterone assays were 9% and 7,5%; 15.5% and 9%, respectively. The TTT kit was designed for human samples and the corticosterone kit for rats and mice.

The antibody for testosterone showed cross-reactivity to dihydrotestosterone (DHT) at 18.75%; for 5α–androstan–3α and 17β–diol it was 3.0%; and for 5–androstan–3β, 17β–diol it was 1.0%. The cross-reactivity shown by the corticosterone antibody was 2.3% with desoxycorticosterone; for cortisol it was 0.27%; for progesterone it was 0.17%; and for testosterone it was 0.47%. For the estradiol concentration, an antibody modified to increase sensitivity to at least 1.0 ng/ml per tube. The antibody showed cross-reactivity of 15.35% with estrone, for estriol 1.74%, and for equilin 4.2%. The sensitivity of the estradiol test was 0.8 pg./tube and the interassay and intraassay coefficients of variation were 8% and 3%, respectively. These hormones were analyzed in the Laboratory of Hormonal Doses at the Instituto Genese de Análises Científicas (IgAc), São Paulo, SP, Brazil, using a Gamma Wizard type reader, model 1470 [96-well microtitre plate (OptiPlate, Perkin-Elmer Life Science, Boston, MA, USA)] and MultiCalc Software (PerkinElmer, Norwalk, CT, USA). The assay was tested for parallelism and accuracy and validated for this species using standard endocrinology quality-control measures in triplicate.

2.5 Immunohistochemistry

A piece of testicles were fixed in 4% buffered formaldehyde for 24 h up to 15 minutes after collection, dehydrated in alcohol solutions at increasing concentrations (70–100%), and then in xylol for embedding in paraffin wax blocks. Sections of 3 μm were then cut using a Leica RM2165 microtome.

Sections were kept for 3 h in a chamber at 70°C dewaxed in xylol and then in decreasing alcohol concentrations, followed by distilled water. They were then heated to 90°C and immersed in buffered citrate solution in a microwave oven. Endogenous peroxidase activity was blocked using H^2O^2 at 3%, and then protein was blocked with Normal Horse Serum (Vector Laboratories, Burlingame, CA, USA) and the samples were incubated with primary antibodies against P450arom, NADPH reductase, and cytochrome P450c17 for 16 h (**Table 1**).

Negative controls were prepared using IgG isotype antibodies (normal rabbit IgG; Santa Cruz Biotechnologies, Dallas, TX, USA). After incubating with the primary antibody, conjugation was made with a secondary anti-mouse/rabbit antibody Immpress Universal kit (Vector Laboratories) and then a Vectastain® ABC Peroxidase Kit amplifying solution was added (Vector Laboratories). For developing, ImmPACT™ 3, 3-diaminobenzidine (DAB) was used (Vector Laboratories). The samples were counterstained with hematoxylin and then mounted on a slide. Photomicrographs of sections of each testis were taken using a Nikon Optiphot light microscope (Model BX51; Olympus Corp., Tokyo, Japan) at 200X. Micrographs of the testis were used to determine the diameter of the seminiferous tubes using ImageJ software (v. 1.43; Research Services Branch, National Institute of Mental Health, Bethesda, MD, USA).

2.6 Western blotting

Frozen testicular and epididymal tissue samples were broken down using a Polytron PT 3000 KINEMATICA® homogenizer (Brinkmann, Westbury, NY, USA), in hypotonic lysis buffer containing 10 nM TRIS/HCl, 5 nM EDTA, pH 7.4, in the presence of the protease inhibitors benzamidine (20 μg/ml), pepstatin (1 μg/ml), leupeptin (0.5 μg/ml), apoprotein (0.1 μg/ml), and phenylmethanesulphonylfluoride (PMSF; 100 g/ml). The homogenate was centrifuged at 10,000×g for 10 minutes at 4°C and the supernatant transferred to 1.5 ml tubes and stored at −20°C. The total

Antibody	Isotype	Immunoreactivity	Dilution (immunohistochemistry/ western blotting)	Catalog numbers
Aromatase	Polyclonal rabbit IgG	Mouse, rat, human, chicken, cow, pig	1:200/1:1000	Abcam (ab18995)
Cytochrome P450 17A1	Polyclonal rabbit IgG	Rabbit, mouse	1:200/1:1000	Donated by Prof. Dr. Alan J. Conley (UC, Davis, CA, USA)
P450 reductase	Polyclonal rabbit IgG	Mouse, rat, sheep, rabbit, guinea pig, hamster, cow, dog, human, pig, monkey	1:200/1:1000	Abcam (ab13513)

Table 1.
List of primary polyclonal antibodies used for immunohistochemistry and western blotting.

protein concentration of the steroidogenic enzymes was analyzed by the method of Bradford (Protein Assay Kit; Bio-Rad, Hercules, CA, USA), and the means obtained were compared against a standard albumin curve read at 595 nm.

Next, 50 μg aliquots of total proteins were denatured in Laemmli buffer (15%) glycerin, 0.05M Tris, 0.055% bromophenol blue, 9% sodium dodecyl sulfate (SDS) containing 6% beta–mercaptoethanol (1:1) and heated to 95°C for 5 min. The samples were separated electrophoretically in SDS–polyacrylamide gels. After separation, the proteins were transferred to polyvinylidene difluoride membranes (PVDF; Bio-Rad Laboratories, Hercules, CA, USA), at a constant current of 120 mA, for 2 h at 4°C in Tris–HCl buffer (Tris–HCl 12.5 mM glycine mM, 1% SDS, and 20% methanol). After transfer, nonspecific antigenetic sites were blocked by a solution of Tris-buffered saline and Tween 20 detergent (TBS-T; 1% Tween 20) and skim milk (5%). The samples were kept in this solution for 2 h with constant agitation at room temperature (20–25°C). P450arom, P450c17, and NADPH reductase were detected using specific antibodies.

After incubation with primary antibodies, the samples were incubated in secondary antibodies conjugated to horseradish peroxidase (HRP, 1:10,000; IgG anti-mouse/anti-rabbit electrochemiluminescence, ECL kit; Amersham Biosciences/GE Healthcare, Chicago, IL, USA) in TBS-T + 1% albumin for 2 h, with constant agitation at room temperature. As an endogenous control, alkaline phosphatase HRP–anti-mouse conjugate (Santa Cruz Biotechnology, Dallas, TX, USA) was used, diluted at 1:50,000 in blocking solution for 30 min. The signal was detected by adding Fast Red–Naftol (Sigma-Aldrich, St. Louis, MO, USA) for alkaline phosphatase for 2 min. The developing solution was from an ECL kit (Amersham Biosciences). The images were taken using a ChemiDoc MP Imaging System (Bio-Rad Laboratories) and normalized against beta-actin (ACTB; 42 kDa), using Image J software.

2.7 Statistical analyses

Analysis of variance was carried out with the GraphPad InSat program (version 4.00 for Windows; GraphPad Prism Software, San Diego, CA, USA) to obtain the means and standard deviations. The Cramer–von Mises normality test was used to verify

homoscedasticity among the variables, and the means were compared using the Student–Newman–Keuls (SNK) test, because the climatic, biometric, and hormonal variables were unstable with coefficients of variation ranging from 15 to 30%. Correlations between the covariables were assessed using Pearson's test. A partial least-squares model was fitted with two components for cross validation. Significance was assumed at $P < 0.05$.

3. Results

3.1 Endocrinology

The hormonal analyses showed significant differences ($P < 0.01$) between the mean testosterone concentration at the peak of the wet season and dry season, which correlated with a high gonadosomatic index and seminiferous tubule diameter compared with spermatogonial development for the month of March ($r = +0.946$), which coincided with the wet season. For the month of September there was another increase in testosterone concentration coinciding with spermiation in the dry season. Another two events were apparent, one being the increase in corticosterone concentration in the month of June (wet season) and the other being the estradiol concentration in the month of December (in the dry season), a period when the species is dormant in the regions studied (**Figure 3**).

3.2 Immunohistochemistry

There were differences in the immunolocalization and intensity of the P450arom, P450c17, and NADPH-reductase in the testes and epididymides in the wet versus dry seasons. In the testes, immunomarkers were observed in the wet season for P450arom in the Leydig cells, Sertoli cells, and germ cells. P450c17 was immunolocalized in the Leydig cells and NADPH-reductase showed light immunostaining in semen and Sertoli cells. Still in the wet season, the P450arom and P450c17 enzymes showed less intense immunolocalization in the Leydig cells, while immunolocalization was not observed for NADPH-reductase (**Figure 4**).

In the epididymides, strong immunostaining for P450arom was observed in the wet season in the interstitial cells, the efferent duct cells, and the sperm contents. The P450c17 and NADPH-reductase enzymes showed slight immunolocalization intensities in the apical cells. In the dry season, the P450arom enzyme had moderate immunolocalization in the interstitial cells and efferent duct cells. The P450c17 enzyme had moderate immunolocalization in the interstitial cells and the efferent duct cells, and light immunostaining in basal epididymal cells. NADPH-reductase showed strong marking in the interstitial cells and in the interior of the efferent ducts, and weak marking for the basal cells (**Figure 5**). **Table 2** shows the enzymes characterized according to intensity and immunolocalization.

3.3 Western blotting

P450c17, P450arom, and NADPH-reductase, in the testes and epididymides, were detected in the extracted protein in the wet and dry seasons. Results were consistent

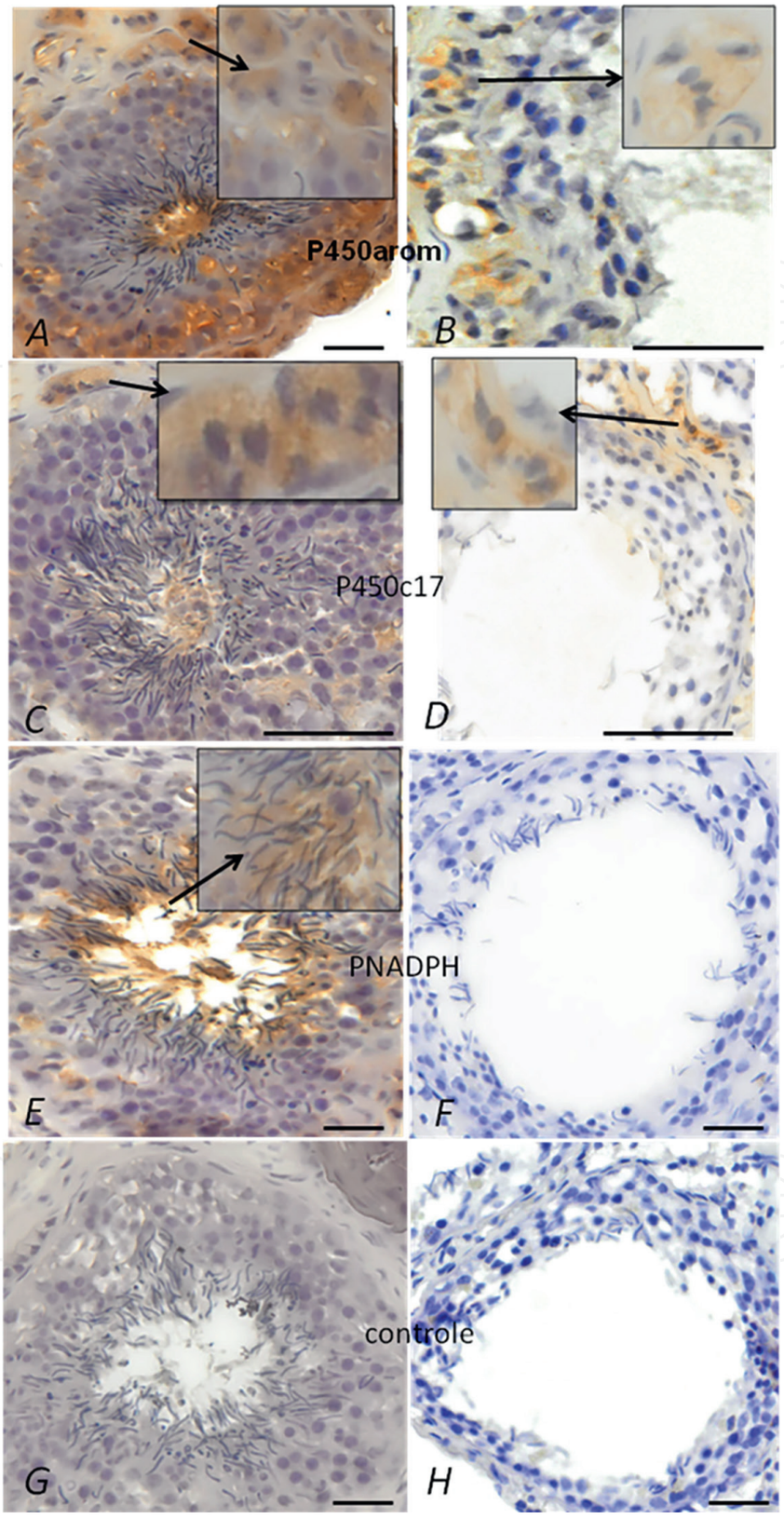

Figure 4.
Immunolocalization of cytochrome P450arom, P450c17 and NADPH-redutase in the K. scorpioides testis in the wet and dry seasons. Negative control (G and H). A, E, F, G, H (Bar 20 μm); B, C, D (Bar 50 μm).

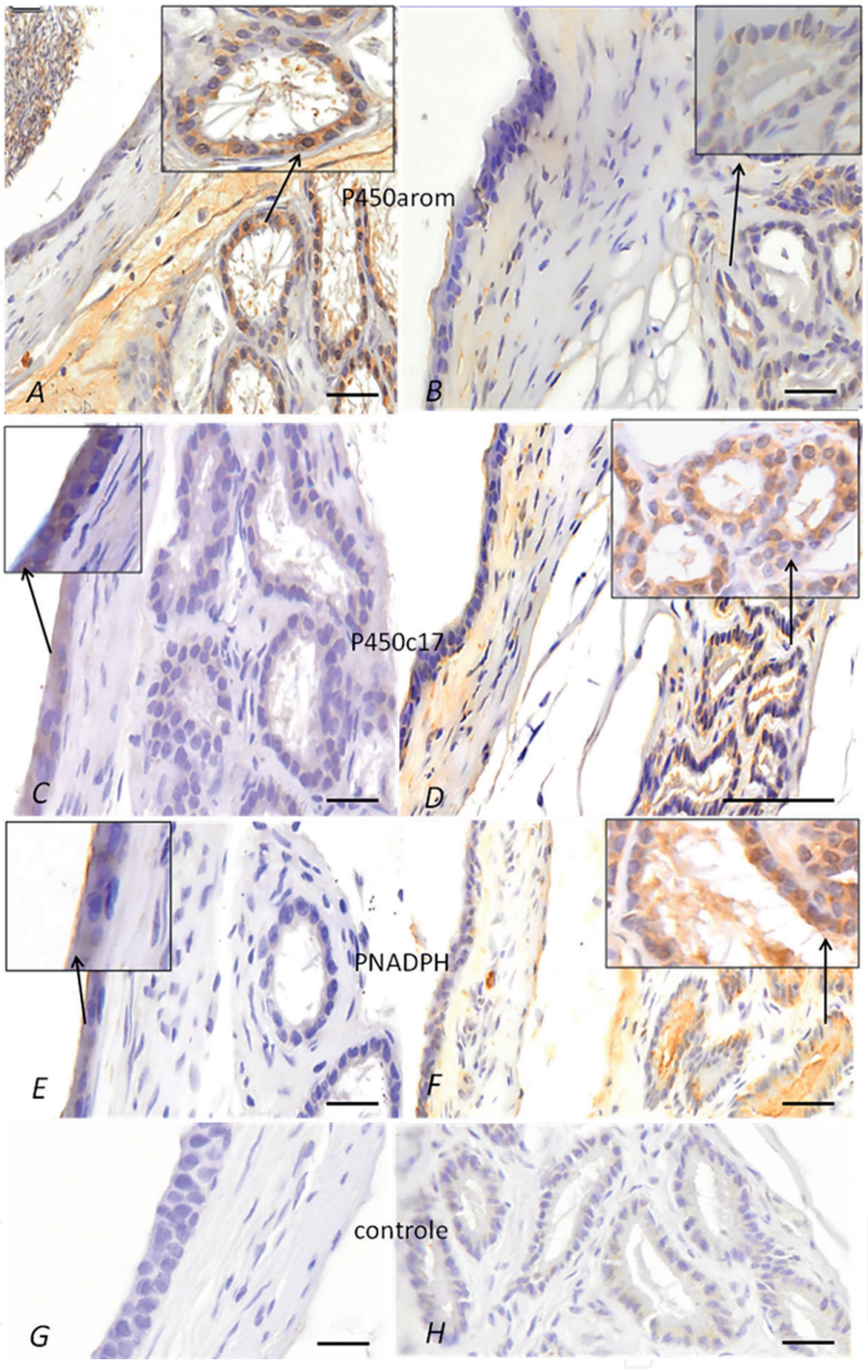

Figure 5.
Immunolocalization of cytochrome P450arom, P450c17 e NADPH-redutase nos epididymis de K. scorpioides in the wet and dry seasons. Negative control (G e H). A, B, C, E, F, G, H (Barra 20 μm); D (Barra 50 μm).

with the immunohistochemical data. Relative expression levels were normalized against beta-actin (42 kDa) for all proteins. Signals were detected with the antibody for NADPH-reductase in the testes and epididymides at 37 kDa, while there was positivity for P450c17 at 57 kDa and for P450 aromatase at 59 kDa for both the wet and dry seasons (**Figure 6**).

Season	Specimens (*n*)	Testis			Epididymis		
		P450	P450c17	NAHPH	P450arom	P450c17	NAHPH
Wet	20	Cl +++, Cs +++, Se++	Cl++, Cs+	Cs+, Se+	Ci +++, De +++, Se +++	Ca +	Ca+
Dry	18	Cl+	Cl++	—	Ci ++, De ++	Ci ++, De++, Cb+	Ci+++, De+++, Cb+

Key: CI, Leydig cells; Cs, Sertoli cells; Se, Semen; Ci, Interstitial cells; De, Efferent duct; Ca, Apical cells; Cb Basal cells; +++/++/+ and – indicate strong/moderate/slight and no immunoreactivity, respectively.

Table 2.
Immunolocalization of the cytochrome enzymes P450arom, P450c17, and NADPH-reductase in the testes and epididymides of K. scorpioides in the wet and dry seasons.

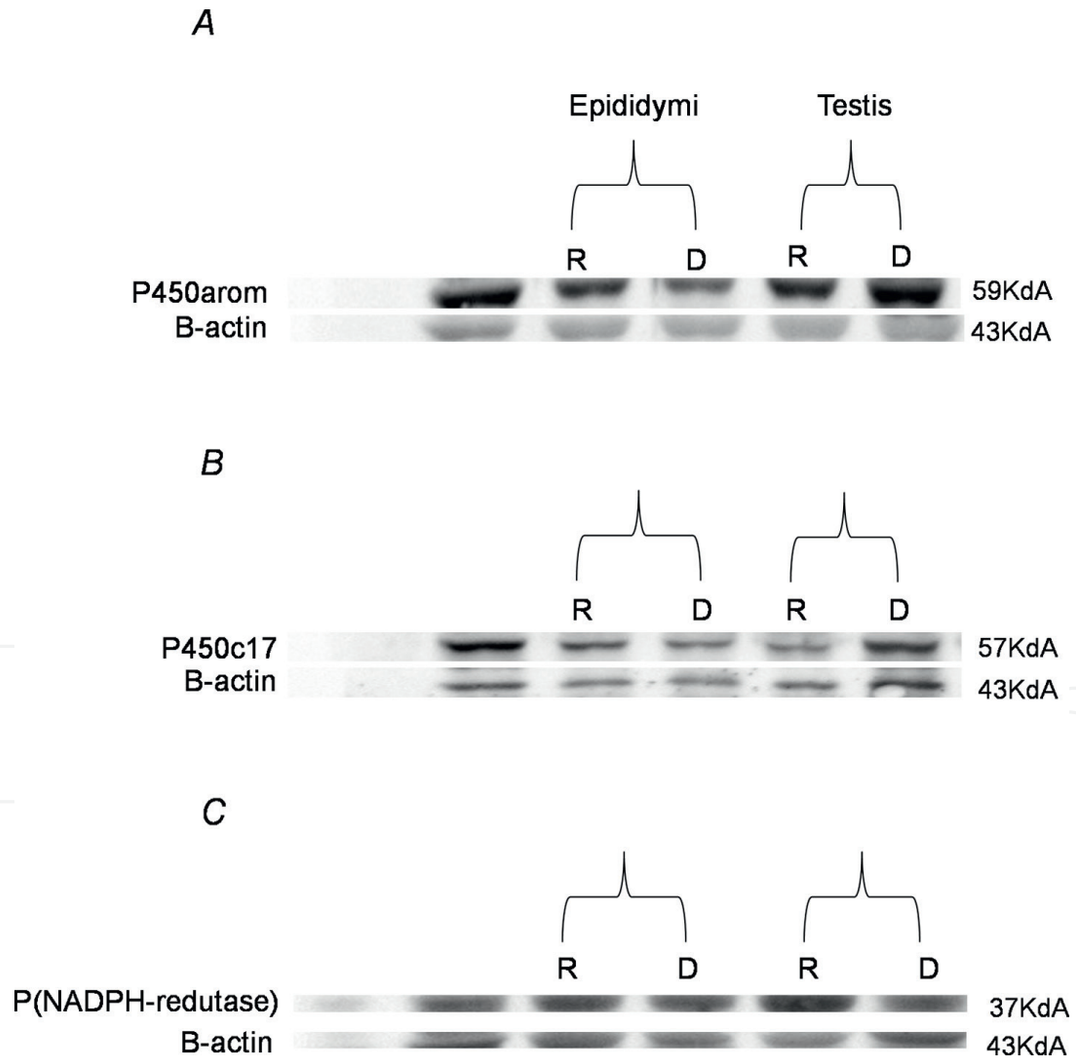

Figure 6.
Expression of the proteins P450arom (A), P450c17 (B) and NADPH-redutase (C) in the testis and epididymes of K. scorpioides turtles. M (marker – Actin beta); Ed (epididymis – Dry season); Er (epididymis – Wet season) td (testis – Dry season); Tr (testis – Wet season).

4. Discussion

The information contained in the literature on the scorpion mud turtle with respect to the seasonal morphological aspects of the male reproductive organs is incomplete. As such, this discussion contains references related to the reptile orders and other classes of animals.

The reproductives parameters analyzed were significantly differed when compare animal from wet and dry seasons. Western blotting showed that these enzymes were also present in both season, however, the differences were observed in cell immunolocalization of the P450arom, P450c17, and NADPH-reductase enzymes in the testes and epididymides. The morphometrics date to adult male animals are considered in turtle with a carapace over 10 cm long [17, 20]. The testosterone hormone profile was associated with the mating season, and the peak spermatogenic activity occurred during the rainy season, coinciding with lower testosterone concentrations compared to the dry season. Observations of the reproductive behavior indicated that seasonal changes in gonad size, testes morphology, and hormone levels all pointed to the influence of environmental conditions in the natural habitat of the scorpion mud turtle on reproductive seasonality [15].

Testes collected in the dry season were golden yellow, as noted by [17] and reported in studies by [21, 22], who described the shape and coloring of the testes of birds and turtle *Kinosternon Scorpioides*. However, they did not highlight changes in coloring linked to reproductive activity or season. These observations are similar to the data obtained by [13, 20] for turtle *Kinosternon scorpioides* in their natural environment. Another interesting fact, also observed by [12], concerns the testis mass, which was greater in the wet season. This suggests that active spermatogenesis occurs in this period.

The mean epididymal mass (0.24 ± 0.09 g) in the dry season, which can be explained by the presence of spermatozoa in the lumen. Although, not as frequent as in the wet season, at this time some animals were seen mating. Furthermore, at the end of the dry period, during the transition to the wet season, the epididymal weight and tubular diameter increased gradually with (0.37 ± 0,27 g) in wet season. That suggests that the animals were preparing for mating [14]. This was also reflected in the morphology of the vas deferens [23]. The analyses of epididymis showed a single elongated fusiform structure, inserted on the dorsomedial surface of the testes, as also described in birds [24, 25].

Immunoreactivities for aromatases in the testis were found in both the Sertoli and Leydig cells, but for the two seasons these immunomarkers were absent in the germ cells and there were differences in intensity. There were less intense reactions in the Leydig cells and weak reactions in the Sertoli cells when the testis was quiescent.

Spermatogonial proliferation was associated with reduced immunoreactivity for the P450arom, which correlated with the development of spermatogenesis. The Sertoli cell intensity decreased during the meiotic phase of the spermatogenesis cycle and returned during the spermiogenesis phase. A similar pattern of immunoreactivity was observed in the Leydig cells. Leydig cells increased in volume during testicular quiescence and showed greater immunoreactivity during the maturation phase of the spermatogenesis cycle. [11] observed immunolocalization of aromatases in the turtle *T. scripta*, but unlike the findings in the present study, they were more intense in the reproductively quiescent period. Insofar as the presence of the enzymes P450arom and P450c17 enzymes could predict hormone synthesis, it seems that estrogen production continued throughout the year, except during the meiotic phase of spermatogenesis. Aromatase enzymatic immunomarking in turtle testes suggests that estrogen

might interfere with spermatogenesis. Estrogen is increasingly studied in the control of spermatogonial proliferation in the testes of amphibians and reptiles because it induces mitotic divisions in the spermatogonium [26].

The immunolocalization of the P450c17 was positive in the testes both season. Androgens produced by Leydig cells play an important role in regulating spermatogenesis and maintenance, such as converting round spermatids to elongated spermatids and cell apoptosis which prevents androgen dependence in spermatogenic tissues [27, 28].

The epididymis of the turtle is not regionally differentiated as in mammals [29], but both types of enzymes were seen in the apical and base cells. The turtle has an uncommon reproductive cycle, in which spermatozoa produced in the wet season are stored in the epididymis for use in the next mating season [30], a characteristic that can be suggested for the species of the present study. The sperm are kept in the epididymis over the year, and during this time sperm motility characteristics are maintained [30].

The data presented can be understood by according to [31], who postulated the existence of partitioning of the enzymes involved in sex steroid synthesis and that in certain compartments there might be effects on estrogen production. In the present study, for example, the epididymis in the wet season showed immunostaining in the apical cells while in the dry season, immunostaining was restricted to the basal cells for P450c17, a fact that might have influenced sperm storage, a common aspect of reproductive physiology of these animals.

The phenomenon of "estivation" is similar to hibernation, which occurs in some animal species. According to [32] animals can stay in this state for weeks or even months in a very cold environment, and [33] observed that estivation can last for up to 6 months for *Kinosternon flavescens*. Because of the high temperatures in our study area, the turtler fresh exhibited dormancy, or estivation behavior ("summer sleep"). This happen when food is scarce, and it is a period unfavorable for survival of the species in the natural environment. [34] observed mating of *K. scorpioides* in captivity in northeastern Brazil, and the greatest incidence occurred during the wet season. This was also observed by [5] of *Kinosternon scorpioides cruentatum* in Mexico. The wet season, from a biological point of view, is a characteristic time for mating, often with more than one female [15, 35].

Lepidochelys kempi in the Caribbean and *Pelodiscus sinensis* in South China show high testosterone levels of 8.44 ng/ml before mating. However, these levels fall during the mating period to 0.65 ng/ml, with intensive spermatogenesis in the hottest season of the year [36, 37]. For the month of September, the season preceding winter hibernation, the plasma testosterone level observed was low at 0.3 ng/ml in *Chrysemys picta* [38]. *Gopherus polyphemus,* a tortoise from southeast Georgia, showed a testosterone peak in July and August that remained high until October, coinciding with mating and spermatogenesis, respectively [39].

When studying the testosterone levels in male *Sceloporus jarrovi* lizards, [40] observed that they were lower in the winter and higher during the spring reproductive period, when the males show high intensity territorial defense behavior. Andò et al. [41] studied the lizard *Podarcis sicula* and observed a peak of 174.8 ng/ml of testosterone in the reproductive period.

The corticosterone concentration for the turtles in our study indicate situations; first, because high values are characteristic of the species, and second, because of the possibly stressful net capture method through the use of fishing net. This method, the same used for fish, might have been the cause, because some studies use different capture methods, such as for *Graptemys flavimaculata*, where a type of handheld net basket was used for capture, and the concentration was only 7 ng/ml in the summer reproductive season [42].

5. Conclusion

Changes in biometric, cell morphometric, and testicular steroidogenesis between the wet and dry seasons suggest that estrogen produced by the Leydig cells can affect germ cell production and apoptosis during spermatogenesis, and the presence of the P450arom and P450c17 enzymes in Leydig cells along with testis recrudescence, also reinforce the idea that they might play a role in testis quiescence. Here, we found that the P450c17, and NADPH-reductase are present in the testis and epididymis at different climatic seasons and might be related to testosterone and estrogen synthesis in turtles. The differences in biometry and spermiogenesis in the wet and dry seasons lead us to support a state of quiescence during the dry period and active spermatogenesis in the wet season.

Acknowledgements

We thank Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) for providing a fellowship to D.C. Viana and FAPEMA for all the extra support provided. Our appreciation to colleague John B. Iverson, who graciously reviewed individual species accounts and offered suggestions for improvements.

Conflict of interest

The authors declare no competing or financial interests.

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