Stages of Embryonic Development of the Amazonian turtle *Kinosternon Scorpioides* (Testudines, Kinosternidae)

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Abstract

Background: The developmental biology of *K. scorpioides* is described, based on the phenotype. This species is important for the flora because they are excellent seed disseminators. In addition, basic embryological information is not yet fully clarified, and this research provides unprecedented information on the chelonian embryology of the Amazonian fauna.

Methods: Females were monitored throughout the reproductive cycle, by video monitoring, to identify nests and the presence of newly laid eggs. At regular weekly intervals, embryo samples were collected fixed in a 4% paraformol solution and preserved in 70% alcohol. For the embryonic characterization we used a stereomicroscope and the scanning electron microscopy method.

Results: We describe 15 embryonic stages for a 15-week (105-day) incubation cycle. Only at 42 days (6th week) was the morphological characterization of a chelonian observed and at the 12th week (Stage XII) the phenotypic characterization of the species kinosternon scorpioides.

Conclusions: In view of the evidence, we found that these phases are similar to the other turtles, with structural variations in the appearance and disappearance of structures due to the specific characteristics for the species.

Background

Investigations about the reproduction of turtles are essential to ensure sustainability, conservation and establishment of reproductive management plans. Despite the longevity of the scorpion mud turtle, tortoises and freshwater and saltwater turtles, they still show low growth rates, which can cause population decline and even extinction due to hunting, deforestation and environmental changes, since they precede hatching, and critical moments during the natural incubation of eggs. These factors do not favor maturity at birth, causing an increase in mortality, reducing the recruitment of young generation to maintain the population [1].

The establishment of embryonic stages in reptiles is defined according to the appearance of external morphological characteristics during development. These being considered requirements for the differential recognition of each stage [2]. Factors such as temperature, humidity and oxygenation are important variables in the embryogenesis of chelonians, as they directly influence the rate of development, incubation duration, size of the cubs, metabolism, behavior and especially sexual determination [3, 4, 5, 6, 7, 8, 9].

Some species of the Testudines order had their embryonic stages determined *Chelydra serpentina* [8], *Lepidochelys olivacea* [10], *Pelodiscus sinensis* [11], *Trachemys scripta* [12], *Emydura subglobosa* [13], *Podocnemis expansa* [14]. However, the species *K. scorpioides* has an unprecedented biological description of the stages of embryonic development.

With a wide geographical distribution, *K. scopioides* is a semi-aquatic turtle, from the Kinosternidae family, a member of the Amazonian wild fauna. It is present in the north and northeast regions of Brazil, in the states of Amazonas, Pará, Amapá, Rondônia, Mato Grosso, Maranhão, Tocantins, Goiás and Minas Gerais [15]. Biological data on this species has been investigated for years by scholars from Maranhão, mainly on its reproductive biology [16, 17, 18].

The importance that the species has for the Amazonian wild fauna, associated with embryological information in a context of rational exploration, supports this study, which aims to describe the embryonic stages of the species, based on the phenotypic description, to serve as a reference in the standardization of each embryological event, to provide an understanding of reproductive management in the elaboration of efficient protection measures in a natural environment, as well as the development of projects to encourage captive breeding for zootechnical and commercial purposes supported by environmental laws.

Methods

2.1 Animals

The animals belong to the Experimental Breeding Ground for the species *Kinosternon scorpioides* (License IBAMA N ° 1899339/2008), in São Luís-MA (02º 31 '47 ”S 44 ° 18' 10" W). A batch of 19 females and 7 males, sexually mature adults (age over 3 years and average carapace length of 14 cm) were monitored by video. Observations in loco daily for the identification of copulation, nesting and laying, in addition to radiographic and ultrasound monitoring of the females were performed, for identify the presence of eggs in the oviducts. All handling was authorized by the Chico Mendes Institute for Biodiversity Conservation (CMIBio) / Brazilian Institute for the Environment and Renewable Resources (IBAMA-MA) with Biodiversity Authorization and Information System (SISBIO) licensing for "in situ" collection of animals under nº 47635-4 / 2016 and also by the Animal Ethics and Experimentation Committee (AEEC) of State University of Maranhão (UEMA) under nº 20/2016.

2.2 Collection of eggs and embryos

Egg samples, after laying, for 12 months, were stored in artificial incubators for reptiles (Jaeger, Germany) with average temperature control at 28 °C. Embryonic development was described from five specimens of eggs, at regular intervals of 1 week, totaling 75 samples. Based on the science of Animal Welfare, embryos, after 21 days, were euthanized with infiltrate directly into the egg with sodium thiopental solution (20 mg/kg) [20], while those in the stages above (56 days) were administered, the same anesthetic in the celomatic cavity (100 mg / kg) [21].

2.3 Description of the embryonic stages

For the description of embryonic development, we based on the appearance of external morphological characteristics, observed through the stereomicroscope (LEICA EZ4) and the images recorded with a

camera (Sony DSC Modelo, 16.2 Mega pixels). The embryos collected were fixed in Paraformol 4% for a period of 48 hours, conserved in 70% alcohol and dehydrated in increasing series of alcohol (80, 90 and 100%), dried in a CO2 oven (24 ° C), glued in support (stub). Sequentially the metal coating with gold by - sputtering and observed in the scanning microscopy (scanning electron microscope (Zeiss LEO 435VP, Cambridge, UK). device for analysis and characterization of the structures.

Result

The average incubation period for the species *Kinosternon scorpioides*, submitted to a controlled temperature of 28 ° C was 105 days (15 weeks) with identification of 15 stages of development, based on the studies of Ytema (1968) for the turtle *Chelydra serpentine*. The descriptions of this study were based on the morphological characteristics of the formation of the eye, mandibular process, limbs and the dermal shield, which served as a basis for comparison with other species of aquatic chelonians described in specific literature (Table 1).

Table 1
List of freshwater chelonian with determined embryonic stages.

Espécies	Nome Comum	Referência
Chelydra		
Chelydra serpentina	Snapping turtle	Ytema (1968)
Chelidae		
Emydura subglobosa	Red-bellied Short-necked turtle	Werneburg, Hugi, Muller e Sánchez-Villagra (2009)
Emydidae		
Chrysemys picta	Painted turtle	Mahmoud, Hess e Klicka (1973) Cordero e Janzen (2013)
Emididae		
Trachemys scripta elegans	Red-eared slider turtle	Greebaum (2002)
Geoemydidae		
Mauremys japonica	Japanese pond turtle	Okada, Yabe e Odas (2011)
Podocnemididae		
Podocnemis unifillis	Yellow-spotted Amazon river turtle	Guzman e Bonilla (1990)
Podocnemis expansa	Arrau turtle	Magalhães (2017)
Trionychidae		
Apolone spinifera	Softshell turtle	Greebaum e Carr (2002)
Pelodicus sinensis	Spiny softshell turtle	Tokida e Kuratani (2001)

3.1 Head and axial development

We characterized Stage I (7 days) by the formation of the embryonic disc, calf and albumen (Fig. 1A), at this stage the embryo was not visible. From Stage II (14 days), the organization of tissues in the formation of the systems was noticed, showing the primitive heart and the formation of blood vessels, and the appearance of cerebral and optical vesicles, pharyngeal arches and curvature of the spine (Fig. 1B and 4A). In Stage IV (28 days), body flexion was evident and continuous, with the embryo looking like an arch, with the formation of the head and cephalic region with four cerebral vesicles, and the beginning of the organization of the nervous system. The optic vesicle defined with pigmented retina, choroidal fissure and pupil (Fig. 1D). In Stage V (35 days), the formation of the head with a well-defined optical placode with a completely pigmented retina and iris and a choroidal fissure like a line is notable,

in addition to the maxillary process extending close to the eyes and fused to the nasal-front process. In *K. scorpioides* the lower eyelid appeared in Stage VI (42 days), while the upper eyelid appeared in Stage VII (49 days), being formed and covering most of the eye in Stage IX (63 days) (1G, 2A, 3D and 4G), we emphasize that the vision organs gain notoriety. The formation of the caruncle, a structure that helps in the opening of the eggshell, is a determining process for its hatching. It is observed in Stage VIII (56 days), when the jaw and mandible are formed and remains until Stage XV (105 days / birth) (Fig. 4F and I). The dermal shields of the K. scorpioides carapace and plastron are fully formed in the final third of development, after 70 days (Stage X), completely showing the characteristic of the species. These structures will form the protective exoskeleton of the viscera (Fig. 2B). The color of the carapace at first is strongly dark, with spots slightly yellowed at the edges with black plastron in the center and slightly yellowed at the ends (Fig. 2E and F).

3.2 Appendicular development

The appearance of the shoots to originate the thoracic and pelvic members occurred simultaneously in Stage III (21 days), and in Stage IV (28 days) the shoots of the thoracic members were larger than the pelvic ones (Fig. 1C and D, 3A and 4B). In Stage V (35 days) a delimitation in the trunk, through a longitudinal groove shows the beginning of the carapace formation (Fig. 1E, 3B and 4C), being well defined in Stage VI (42 days), and still, in continuous growth and marked characteristic of the limbs and skin pigmentation, appearance of digital plates showing the peculiar shape of the Chelonia order (Fig. 1F, 3C and 4D). At 42 days (Stage VI) the members have digital plates, at 49 days (Stage VII) there are digital plates and the digits forming a serrated aspect of the members' edges, at 56 days (Stage VIII) the five digits are separated by interdigital membranes and at 70 (Stage X) and 98 days (Stage XIV), they are formed with claws, skinfolds and scales (Fig. 3C-3I).

3.3 Macroscopic sexual differentiation

The urogenital papilla, a structure that changes to sexual differentiation, was observed after 56 days (Stage VIII), beginning to be surrounded by the cloacal structure at 63 days (Stage IX), with the cloaca in formation at 84 days (Stage XII) Figure (1H, 2A and D). The tail is short, and after 70 days (Stage X) the formation of a corneal appendix at the extremity is observed, which gives the name to the species, due to the similarity to the scorpion's tail. At 105 days of incubation (Stage XV) hatching occurred with the birth of the cubs. At this stage the phenotypic pattern of the species is very marking, however, there is still no differentiation from sexual dimorphism. All babies were similar, with a dark gray triangular head with yellow spots and a well defined elongated snout. Neck with gray or black skinfolds. Presence of three pairs of mentonian barbels. The carapace has thirty-six dermal plates (nuchal, vertebral, costal and marginal) while the plastron is outerally covered by six pairs of corneal shields called gular, humeral, pectoral, abdominal, femoral and anal. Thoracic and pelvic members with reduced interdigital membranes with five fingers with pointed nails. Vitellinic peduncle closed and marked by a central ring in the plastron and a small vitelline bag may be persistent (Fig. 2G). The tail is the same size for all newly

hatched, not being a differentiation item, as occurs in adults, where the male has a long tail in relation to the female.

Discussion

The embryos of *K. scorpioides* can be distinguished from other species by their phenotypic pattern of pigmentation, characteristics of the longitudinal keel of the carapace, dermal shields of the exoskeleton, formed by the junction of the carapace with the plastron. Comparisons on the development between chelonian species is still complex, due to differences in size and time involved in embryonic phases [22]. The use of measures of body size, chronological age and morphological stage are tools used to standardize these interspecific comparisons [23]. Traditionally, two “test models” have been used for Testudines, those of [6, 10]. They serve as a basis for studies that have described the embryology of turtle development, and there are differences in development between species.

The appearance of the chelonian carapace is a variable element between species, both in the stages of development, as well as in morphology and color. At 42 days (Stage VI) the carapace of the *K. scorpioides* embryo is defined, with the beginning of the formation of the plastron, in a characteristic pattern of the turtles. These findings are consistent with other species of turtles, such as the marine *Chrysemys picta* and *Chelonia mydas*, in studies similar to this one, appearing between 22 and 28 days of incubation, which is shorter, but assigned to the shorter incubation time [24].

The initial formation of the eyes is initiated by the appearance of the optic vesicle, highlighting the visual apparatus in a marked way after the development of the retina, iris, pupil and lower and upper eyelids. The optic vesicle is clearly visible in *P. expansa* in stage 6 (6 days), while the optic vesicle is visible in stage 7 (9 days) in *Mauremys japonica* [9] and C. *serpentina* [10] in stage 8 (4 days) in *Pelodiscus sinensis* [12].

In the red turtle (*Emydura subglobosa*), the lower eyelid appears early, around the 13th day (stage 5) and already begins to overlap the eye, reaching the lens level [13]. In this aspect, in the *C. serpentina* turtle these characteristics were observed at 30 days of development and at 20 days in *P. sinensis* [10, 12]. In *M. japonica* (stone turtle), the caruncle was initially evidenced as a small white process under the nostril (stage 17), showing a sharp point in stage 26, a peculiar characteristic of the species [9]. At 63 days (Stage IX), the ranfoteca is observed covering the branches of the mandible together with the formation of the barbel in the ventral region of the mandible (Fig. 2A and 4D). It helps animals to select, shatter and ingest their food to replace teeth. It has its own characteristics that vary according to the diet, and can therefore be used to identify different species of turtles [25].

Differently from the description by [8], for *Trachemys scripta*, which was based only on the characteristics of the thoracic members as a criterion for delimiting the stages. In *K. scorpioides* we have deepened the characterization of the various stages of development starting from the initial stage until the hatching. For [8] the morphology of the thoracic limbs is the main criterion for delimiting the stages, as it is easily analyzed until the second half of development, and is highly conserved among most turtles. The

tendency in the development of the limbs is that the thoracic ones precede the pelvic ones, an event described in the species (*Chelydra serpentina*, *Pelodiscus sinensis*, *Emydura subglobosa* and *M. japonica*), or both manifest simultaneously and synchronously (*Podecnemis expansa* and *Chrysemys picta*) [12].

The sexual dimorphism of the adults of *K. scorpioides* is striking, with males showing long tail and concave plastron and females with short tail and straight plastron [26]. In newly hatched cubs the tail is the same size, still short and the plastron straight. Another important structure in sexual differentiation is the urogenital papilla, [14] describes that it is the precursor of the cloaca in the Amazon turtle (*Podocnemis expansa*), appearing at 30–35 days (Stage 19); in *Emydura subglobosa*, this structure becomes noticeable at 28 days (Stage 9) [13].

The growth rates of vertebrate embryos are highly variable within and between species and depend on the incubation temperature [27]. The incubation temperature influences its duration and the degree of development [28], at low temperatures they increase the time and decrease the rate of development, while at high temperature they decrease the duration and increase the rate of development [4, 29, 30, 31]. In this way, these temperatures need to be taken into account when comparing, since eggs incubated at different temperatures can present divergent results. In *K. scorpioides* the eggs were incubated with a controlled average temperature of 28 ° C. The observations, even similar to other reported species, had some important differences and they may be linked to the morphological characters of the interspecific variations, while the incubation temperature influences the differences related to the embryonic stage.

The morphological characteristics of the embryonic development of the species *K. scorpioides* showed similar states, with few differences, to other turtles. Some characteristics were found delayed or accelerated in comparison to the findings of [10], for the turtle *Chelydra serpentina*. In addition, strong evidence from this study, supports the hypothesis that the morphological characteristics of the embryonic development of this species, resemble the other chelonians, presenting some variations in the appearance and disappearance of structures.

The information generated by the analysis of the embryonic development of K. scorpioides provides a basis for research with species of the same genus and also with other species of freshwater tortoises, since the temperature and humidity conditions that the eggs are submitted to during the incubation act in the variation of this period, in the speed of metabolic reactions and consequently in the development of the embryo. Another aspect in the difference between the chronology of development in turtles, can be attributed to the necessary morphological characteristics to each species according to their habitat as a result of the evolution process itself. Thus, the present study contributes to the understanding of the various stages of embryonic evolution and reproductive management of this controlled species in captivity, taking into account the incubation temperature, and it is worth emphasizing the importance of expanding the studies to understand the intraspecific variations.

Conclusion

In *Kinostenon scorpioides*, the stages of embryonic development occurred in 105 days, from laying to hatching, in moments of structural formations similar to other turtles, even at different times. There is a strong influence of the elements temperature and humidity.

Declarations

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Authors' contributions

L.P.F.A. Chaves (execution); D.C.V. (planning and supervision), L.T. (planning and supervision); J.M.A.C. (execution); A.C.A.N. (planning and supervision); A.L.S. (coordination). All authors have read the manuscript and have approved this submission.

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Availability of data and materials

The datasets analysed during the current study are available from thecorresponding author on reasonable request.

Ethics approval and Consent to participate

All handling was authorized by the Chico Mendes Institute for Biodiversity Conservation (CMIBio) / Brazilian Institute for the Environment and Renewable Resources (IBAMA-MA) with Biodiversity Authorization and Information System (SISBIO) licensing for "in situ" collection of animals under nº 47635-4 / 2016 and also by the Animal Ethics and Experimentation Committee (AEEC) of State University of Maranhão (UEMA) under nº 20/2016.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figures

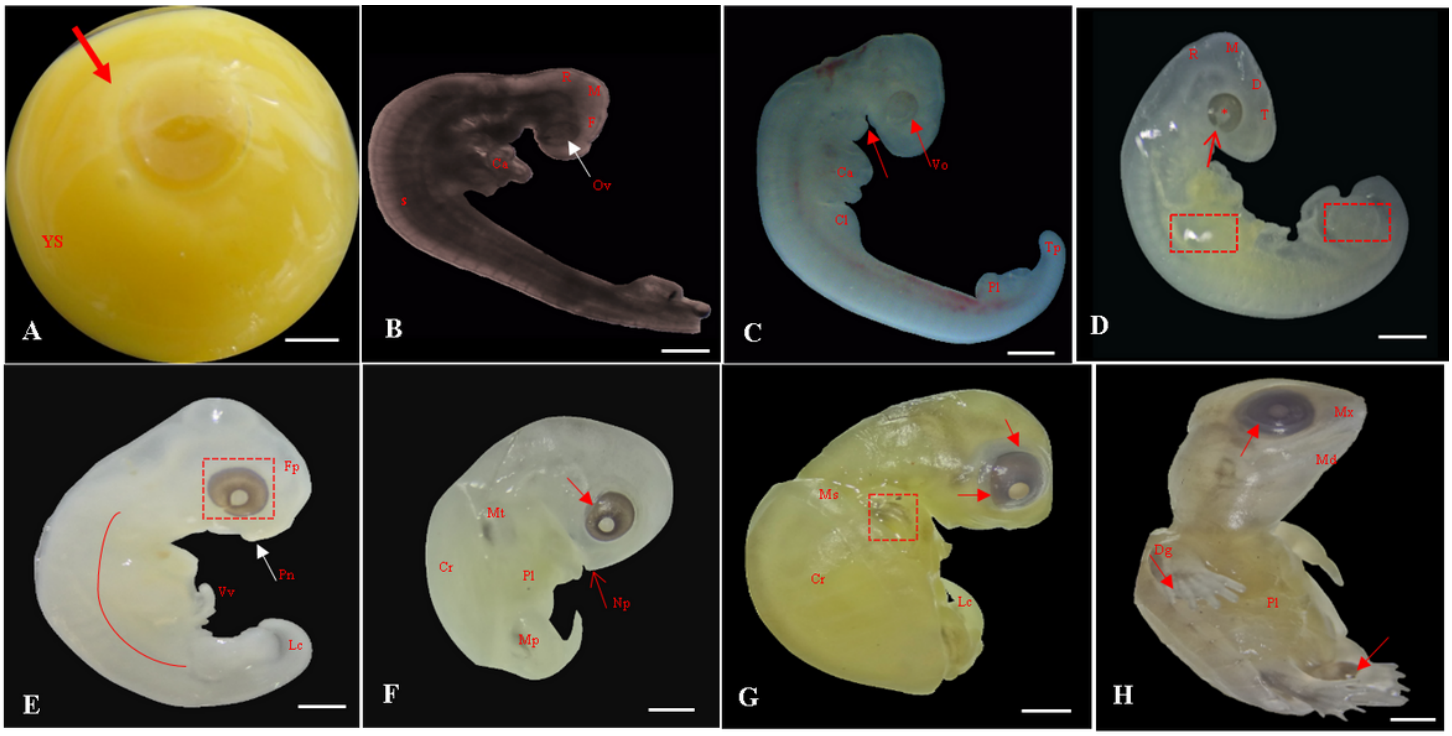

Figure 1

Embryonic development of Kinosternon scorpioides. Side view: A - Stage I: Yolk sac (YS), Blastodisc (arrow); B- Stage II: Cerebral vesicles - Forebrain (F), Midbrain (M), Rhombencephalon (R), Optic vesicle (Ov), Somites (S), Cardiac area (Ca). C- Stage III: Pharyngeal arch (arrow), Cardiac area (Ca), Chest limb bud (Cl), Pelvic limb bud (Pl), Tail process (Tp). D- Stage IV: Cerebral vesicles - Telencephalon (T), Diencephalon (D), Midbrain (M), Rombencephalon (R), Pupil (asterisk), Choroidal fissure (arrow). E - Stage V: Placode (dashed rectangle), Fronto-nasal process (Fp), Longitudinal groove (red line), Vitelline vein (Vv). F- Stage VI: Nasal pit (Np), Carapace (Cr), Pastron in formation (Pl), upper eyelid (arrow); G- Stage VII: Marginal shields (Ms), Pigmentation of the base of the digits (dashed rectangle), upper and lower eyelid (arrow); H- stage VIII: Maxilla (Mx), Mandible (Md), Interdigital membrane (arrow), Urogenital papilla (Up). * Image of Kinosternon scorpioides embryos in different stages of incubation. Barr scale: 0.1 cm.

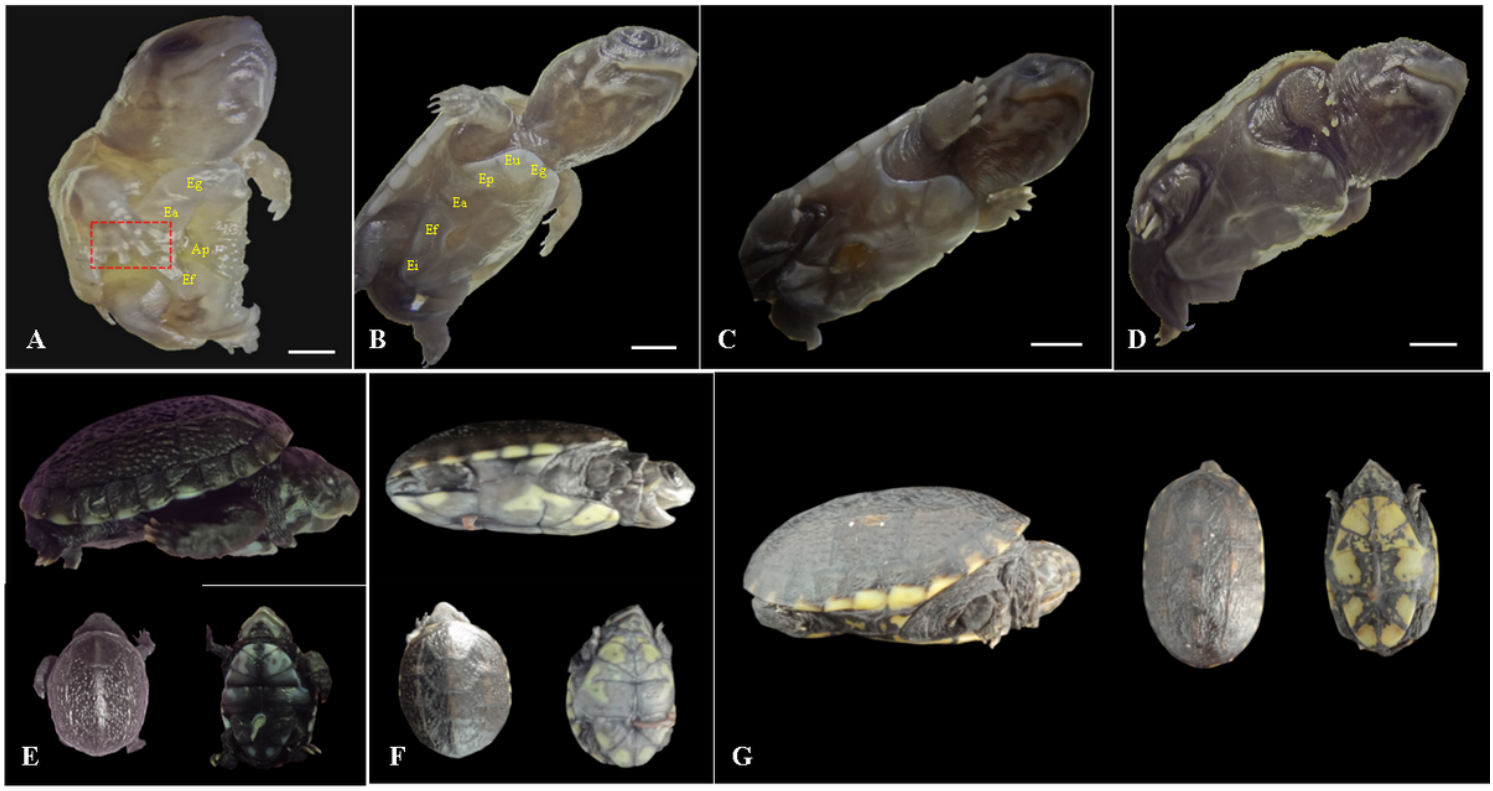

Figure 2

Embryonic development of Kinosternon scorpioides. Side view: A - Stage IX: Abdominal shield (As), Gular shield (Gs) and Femoral shield (Fs), Opening of the plastron (Op); B- Stage X: Dermal shields: Gular (G), Humeral (H), Abdominal (A), Femoral (F), Axillary (Ax), Inguinal shield (I). C- Stage XI: Head is light gray with small white spots and claws are thick and curved; D- Stage XII: Neck and limbs take on a dark gray color; scales on the limbs and longer claws. Dorsal and ventral view: E, F and G - Stage XIII to XV (day of birth): marked development of the phenotypic patterns of the species. * Image of Kinosternon scorpioides embryos at different stages of incubation. Barrel scale: 0.5 cm.

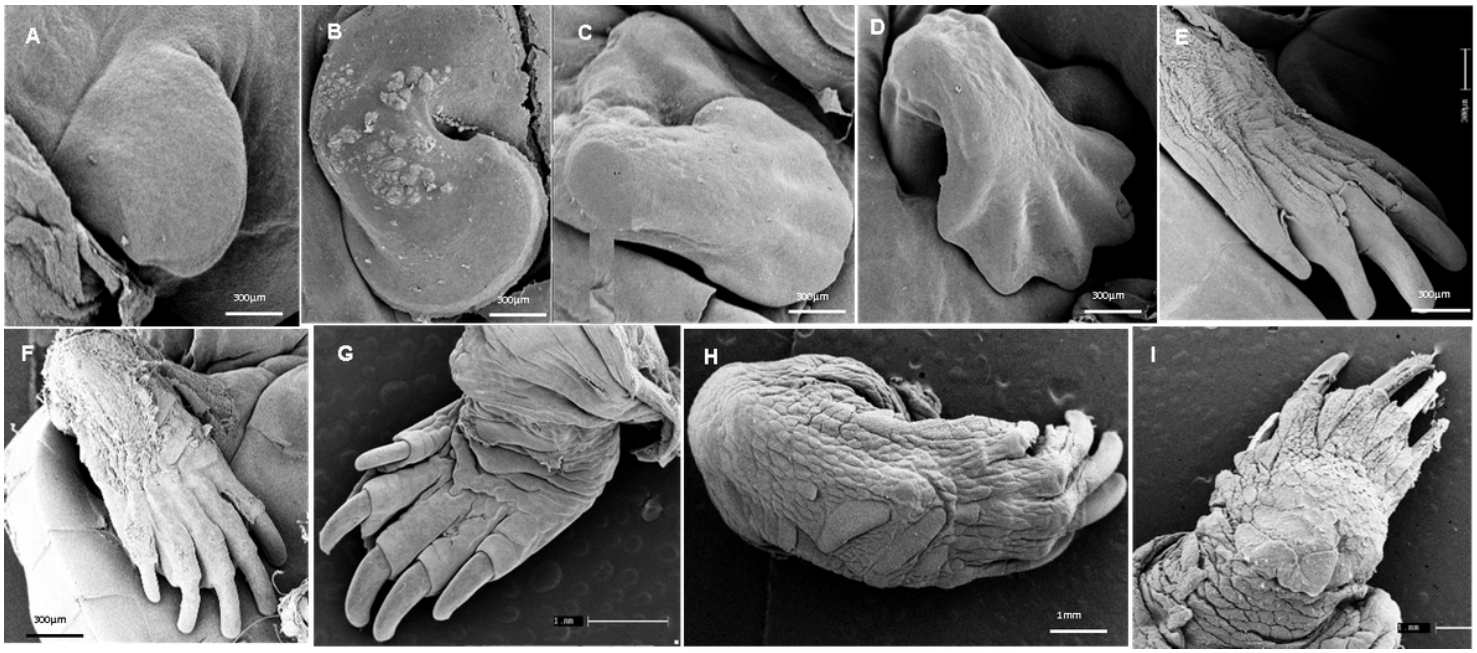

Figure 3

Electromicrograph of the thoracic limbs of Kinosternon scorpioides embryos. A: Stage III - Beginning of bud formation. B: Stage V - Bulging of the shoots in the lateral regions. C: Stage VI - Emergence of digital cards. D: Stage VII- Member with a serrated edge. E: Stage VIII - Five digits separated by digital membranes. F: Stage X- Digits with folded skin and claws; G: Stage XI - Higher number of skin folds, scales and thick and curved claws; H: Stage XII- Higher number of scales. I: Stage XIV- Member fully formed.

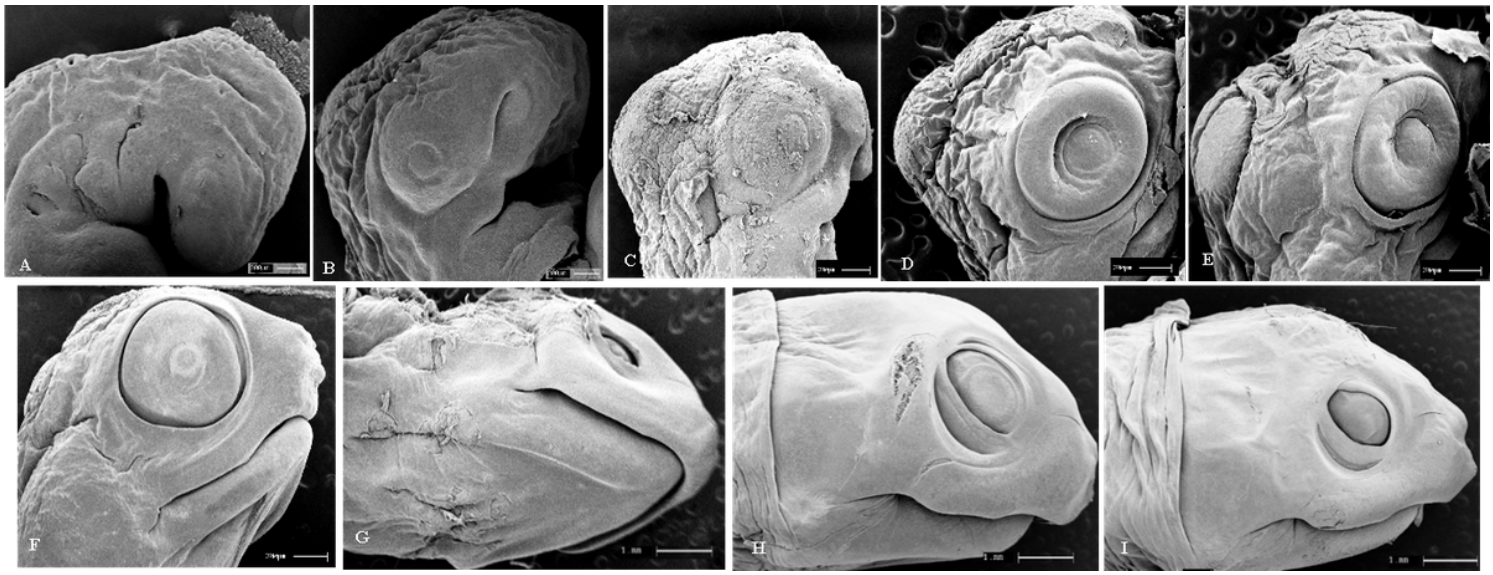

Figure 4

Electromicrograph of the head of Kinosternon scorpioides embryos. A: Stage II - Optic vesicle slightly recognized; pharyngeal arches. B: Stage III - more evident optic vesicle; maxillary and mandibular process. C: Stage V - Head region presents placode formed with retina and iris; maxillary process fused with the

fronto-nasal process. D: Stage VI- Growth of the lower eyelids. E: Stage VII- Prominent placebo and formation of the upper eyelids; F: Stage VIII- Upper and lower eyelids beyond the edge of the eye; fully formed maxilla and mandible; presence of caruncle. G: Stage IX- Upper and lower eyelids; jaw, rhamphoteca and dewlap. H: Stage XI - Projections of the dermal papillae and a greater number of skin folds in the neck. I: Stage XII to Stage XIV - Most prominent and numerous neck folds; fully formed eyelids, clear pupils and presence of caruncle.

Supplementary Files

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- ARRIVEguidelines.docx
- Availabilityofdataandmaterial.docx