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Characteristics of *Kinosternon scorpioides* turtle embryos through egg candling

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A R T I C L E I N F O

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A B S T R A C T

Effective management practices are critical for the successful reproduction of animals in captivity or in *in situ* environments. One of the techniques used in egg quality control is candling. In *ex situ* reproduction of *Kinosternon scorpioides*, it is important to establish incubation protocols, which, through egg management, allow control of embryonic development and improved birth rates. We incubated eggs and monitored embryonic development of *K. scorpioides* through egg candling. Fertile eggs were incubated, and candling analyses were performed weekly. Embryonic development in the early stages (first third of incubation) presented the following characteristics: presence of the vitelline circulatory system, expansion of this system, and descent of the embryo onto the yolk. Then, in the middle stages (second third of incubation), the intense pigmentation of the embryo was observed, with the body moving to prone position. Subsequently, in the late stages, the embryo grew, filling a large part of the internal space of the egg. The incubation period took on average 116.50 ± 13.11 days. Candling can be used to monitor the embryonic development of *K. scorpioides*. It is a useful tool to improve the production of hatchlings, from fertility detection to hatching.

1. Introduction

Captive breeding of chelonians is an aquaculture activity conducted worldwide [1]. Turtles are commonly kept as pets and there are large numbers of species traded annually [2,3]. In Brazil, this practice is still incipient, but the North region of the country carry out management activities of these animals, which could result in a reduced impact on the natural populations [4].

In this sense, *ex situ* chelonian reproduction programs are seen as essential for the maintenance of some species [5,6]. Of note, understanding the conditions for incubation and hatching of chelonian eggs is crucial to improve the hatchling production, contributing to *in situ* and *ex situ* conservation efforts [7,8].

In incubation management, the simplest and most accessible method is called candling, which consists of observing the internal characteristics of eggs using a bright light [9]. This technique, widely used in poultry farming, is considered extremely important when monitoring embryonic development to ensure good hatchlings production [10–12]. In chelonians, it was tested to monitor egg fertility and diapause during the animals' embryonic development [13,14].

In a study of the embryonic development of 37 species of chelonians candling was used to identify five characteristics, which are described below: 1- presence of areas of vascularization; 2- development of the vitelline circulatory system; 3- eye pigmentation; 4- body pigmentation; 5- hatching period [9]. In addition, the embryonic development of *Carettochelys insculpta* was studied of through candling [15], correlating it with characteristics observed during the different morphological stages of the embryo's development: 1- presence of maxillary and mandibular processes; 2- optic capsule; 3- development of limbs and carapace [13,16,17].

Kinosternon scorpioides (Kinosternidae) is widely distributed in Central and South America. It is a species hunted for consumption and trade by local communities [18]. Unsustainable use of this species is leading to a reduction in natural populations [19]. However, *ex situ* management initiatives are being developed to produce this turtle, which exhibits promising aquaculture potential, characterized by good growth and

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reproduction in captivity [20–22]. *K. scorpioides* turtles lay on average two or three eggs [19] and an adult female can lay one to two clutches every year [21].

It is important to understand that studies about the reproductive biology of Amazonian chelonians is required for an efficient development of *ex situ* conservation methods [23]. The egg incubation phase is the most vulnerable period in the chelonian life cycle, thus knowledge about the factors that influence incubation and embryogenesis can improve the conservation and rational management of the species [24,25]. This highlights the importance of understanding the optimal conditions for egg incubation and hatching, which are essential to animal production, without compromising species in the natural environment. Based on this argument, the main objective of this study was incubating eggs and monitor the development of *K. scorpioides* embryos by candling. Furthermore, the embryonic characteristics and incubation stages was observed.

2. Methods

2.1. Scientific breeding

Kinosternon scorpioides belonged to the scientific farm of the Brazilian Agricultural Research Corporation (Embrapa) (LO 7310/2014-SEMAS-PA), located on Marajó Island (Salvaterra, Pará, Brazil, 48°33'23.32"W and 0°42'25.26"S). This study was approved by the Animal Ethics Committee (n° 001/2016) of Embrapa - Eastern Amazon region.

The breeding enclosure had a reproduction area (80 m^2), which had a water tank and a sand area, where eggs were laid. Adults (n = 138 females; n = 58 males), aged over three years, weigh 500 g on average [21]. The animals received commercial food (32 % crude protein), three times a week, based on 1 % of body weight. The clutches (one to five eggs) were collected in 19 nests.

2.2. Sample collection and incubation

Only fertilized eggs were selected, through candling, after seven days of laid. Fertilized eggs were those with the presence of a yolk resting on the bottom of the egg, or presenting an opaque white spot [26,27]. Eggs were collected daily, (7–9 am), and were kept in the same position as the nest [28,29], the eggs were identified using a pencil; subsequently were weighed (0.001 g of precision, Eastdall balance) and measured (length and width), with a pachymeter (0.02 mm scale, Starfer).

The eggs were placed on a plastic tray containing vermiculite substrate (moistened to 80 %) to be transferred to the Ecological Premium® IP-R incubator with digital temperature control (28.5 ± 0.5°C) (Fig. 1). To calculate the amount of water, the substrate was prepared in a kiln [30] and dried at 95–110 °C for 72 h. During the period the experiment the trays were weighed twice a week and, if necessary, water was added [24,31].

2.3. Candling

The eggs were observed, preferably at night, once a week, to monitor embryo development (Fig. 1). A torch light was used for candling [14], and photographs were taken with a camera (CANON® EOS Rebel T5, CANON® macro lens 60 mm). Moreover, a ring to support the egg was used, to prevented rotation and eventual rupture of the extra-embryonic membranes, which would cause embryo death.

The embryonic morphology [24] was used to support candling analyses, and can be observed in Fig. 2. In the early stages of incubation (first third of incubation), 58 eggs were observed by candling; in the middle stages (second third of incubation), 39 eggs were analyzed; and in the last stages, 15 eggs. During the experiment, some eggs were removed from the incubator to study the embryonic histology. Due to this analysis, there was a decrease in the number of eggs observed by candling.

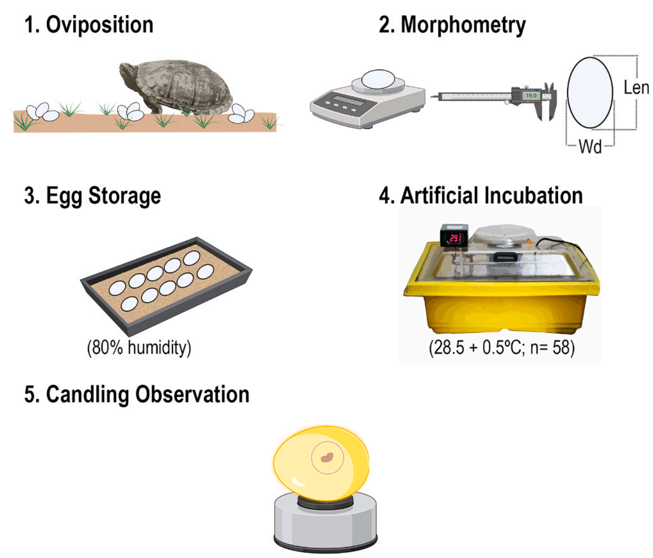

Fig. 1. *Kinosternon scorpioides* incubation management. 1) Eggs to be collected from *K. scorpioides* nest. 2) Measurement of egg morphometry (Len: length, Wd: width). 3) Placement of eggs in containers containing commercial vermiculite at 80 % humidity. 4) Play containers in egg incubator. 5) Candling of the eggs. Image created with Biorender (2023).

In the hatchlings, morphometric variables were measured (n=15), such as the straight length of the carapace and plastron, as well as the width and mass.

2.4. Statistical analysis

Mass (eggs and hatchlings), morphometric data and days of incubation were plotted in spreadsheets (Microsoft Office Excel) to calculate means and standard deviations (n = 58). To evaluate the variances in the eggs mass (n = 15) observed across different developmental stages (early, middle, and late) throughout the incubation period, a Kruskal-Wallis test was employed due to the non-conformity of our data to the assumption of homogeneity. Spearman's correlation analysis was employed to investigate the association between the variables of initial egg mass and hatchlings (n = 15). The R studio ® was used for statistical analyses [32].

3. Results

The results of this study showed that *K. scorpioides* eggs, when laid, are white and ellipsoid (Fig. 3 A, B), and when exposed to light, they are translucent, bright, and yellowish due the high amount of yolk. It should be mentioned that only one egg maintained this appearance until day 40 of the incubation period. At the end of the incubation period, close to hatching (Fig. 3 C), cracks were observed on the eggshells. These cracks were longitudinal and were observed in the peripheral part of the egg (Fig. 3 D).

After one week of oviposition, the eggs were classified as fertile or infertile. Embryonic death was observed by structural deterioration, as following alterations: 1- rotten smell; 2- dark spots outside; 3- rough shell; 4- opaque and shapeless dark spots internally (Fig. 3 E, F). In contrast, our results showed that the fertile eggs that did not present this morphology were kept in the incubator.

3.1. Monitoring embryos by candling

3.1.1. Early stages

The yolk was seen resting inside the shell, from day 7. The first sign of embryonic development was seen between day 15 and 35 of the incubation period, with formation of the vitelline circulatory system

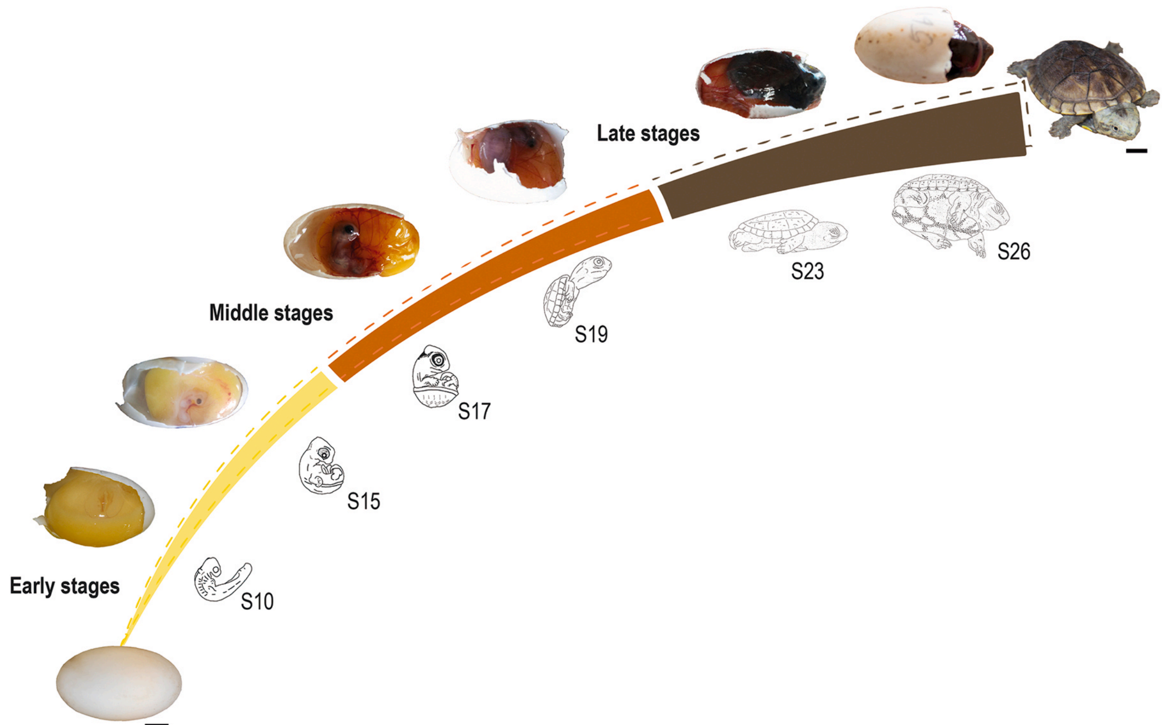

Fig. 2. Embryonic morphology used to support candling analyses (S: embryonic development stages, based in Braga et al., 2021). Scale bar: 1 cm.

which expanded from the yolk (Fig. 4 A and B) (n = 58). In the early period of incubation, the embryos were slightly curved, in the lateral decubitus position, close to the eggshell, in the opposite direction to the yolk. At the end of these stages, the embryo was descending onto the yolk (Fig. 4 C and D). Embryonic deaths were not observed during these stages.

3.1.2. *Middle stages*

The embryo was suspended over the yolk (n = 39), supported by vessels of the vitelline membrane, from day 41 to day 60 of incubation remaining in the lateral decubitus position over the yolk (Fig. 4 E). Some samples presented ocular pigmentation. The embryo length was about two-thirds smaller than the egg size. On day 61, the embryos started to change to the prone position (Fig. 4 F), showing limb movements. The body pigmentation was observed. A single embryonic death was observed during these stages.

3.1.3. *Late stages*

The intensification of body pigmentation did not allow the light to clearly show the structures (Fig. 4 G). The embryo (n = 15) began to fill a large part of the internal space of the egg (Fig. 4 H), showing clear movements of the limbs. As the incubation period progressed, the air chamber can be clearly observed. The size of the yolk decreased, which was internalized at pre-hatching and no longer apparent using the candling technique. Two embryonic deaths were observed in these stages.

In the present study, the length of eggs laid (n = 58) was 3.66 ± 0.18 cm, width was 1.92 ± 0.07 cm, and mass was 8.01 ± 1.09 g. A significant difference was not observed in the weight of eggs (n = 15) when comparing the incubation stages (Kruskal-Wallis = 0.31, df = 2, $p > 0.005$).

The incubation period lasted, on average, 116.50 ± 13.11 days (n=15). The hatchlings (n = 15) had an average straight carapace length of 2.89 ± 0.4 cm and width of 2.00 ± 0.31 cm; the average straight plastron length was 2.42 ± 0.33 cm and width were 1.60 ± 0.09 cm. The height between the carapace and the plastron was 1.70 ± 0.28 cm; and the average mass was 5.19 ± 2.26 g. Moreover, the Spearman's correlation revealed a notable positive correlation (ρ=0.81; $p < 0.005$) between the initial weight of eggs and hatchling mass (n = 15).

4. Discussion

4.1. *Embryonic development by egg candling*

Chelonian incubation management uses an efficient methodology [13], which, through candling, allows monitoring the viability of eggs and the development of embryos. The egg characteristics obtained by candling showed that the embryonic development of *K. scorpioides* is slow when compared to that of *C. insculpta* [15]. During the incubation of *K. scorpioides* eggs, the following were observed: 1- formation of the vitelline circulatory system (between day 15 and 35 of incubation), 2- embryo resting onto the yolk (day 41–60), 3- growth, pigmentation, and 4- change in embryo body position (starting on day 61). For *C. insculpta* eggs (≥ 77 days) these periods occurred from day 8–10, day 29–32, to day 36–41, respectively [15].

The first sign of embryonic development in *K. scorpioides* eggs was the formation of the vitelline circulatory system. However, in a study conducted with *C. insculpta* [15], the embryo development began with an opaque spot on the eggshell, on day 7 of incubation, this spot was possibly related to the egg fertility, subsequently, vascularization areas were observed, which was the initial formation of the vitelline circulatory system [28,33]. Likewise, in *K. scorpioides,* opaque white spots were also noticed, and used as a criterion to define the egg fertilization, as well adopted for sea turtle *Lepidochelys olivacea* [28].

During the early stages, discernible alterations were noted in the internal appearance of *K. scorpioides,* with it becoming possible to observe the presence of the embryo and blood vessels comprising the vitelline circulatory system. The appearance of the haemodisc was observed in the first days of *K. scorpioides* incubation [24], as the same as view in *Pseudemydura umbrina* [34]. In another aspect related to this issue, the vitelline circulatory system of *Macroclemys temminckii* reached regions close to the equatorial sides of the egg [9].

The results of the present study showed that, the period of growth, pigmentation, and change in body position was observed in middles

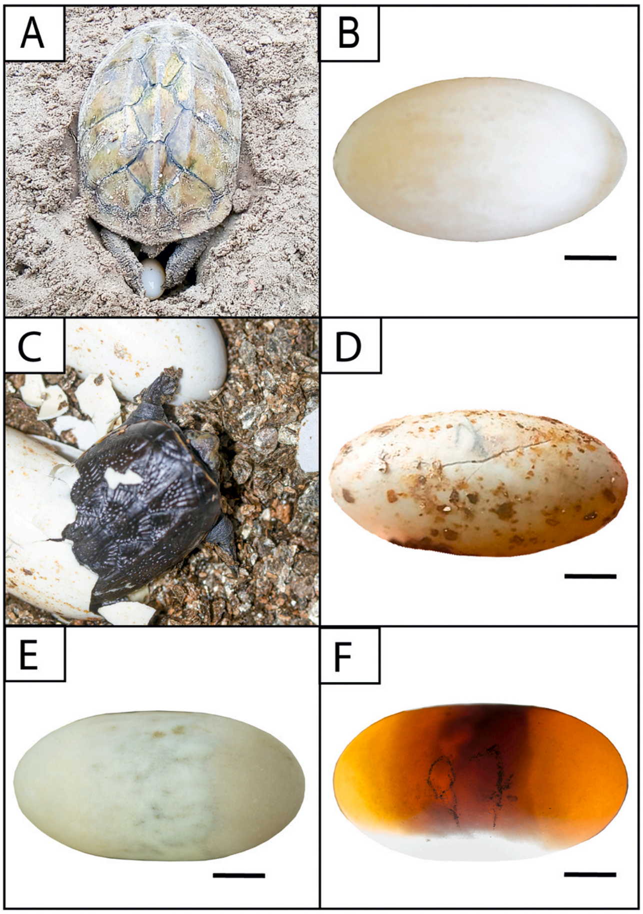

Fig. 3. A) Oviposition of *Kinosternon scorpioides* eggs. B) Newly laid egg; C) Hatching of *K. scorpioides*. D) Egg showing longitudinal cracks, characteristic of the hatching process; E) Macroscopic appearance of a contaminated egg, rough appearance with dark spots; F) Candling showing misshapen dark spots in a contaminated egg. Scale bar: 1 cm.

stages. In *Graptemys pseudogeographica* the embryos (stages 21 and 22, [35]) move their limbs, as formed in this work [9]. The larger size of the amnion, noted at these embryonic stages, allows for greater mobility [9]. In embryos of *C. insculpta* [15], these characteristics were also seen at the end of the development period, where the prone position was already observed, with neck lateralization. In contrast, at the end of incubation, the embryo moved its head in the anterolateral direction [15], which was not observed in the species in our study.

The incubation period, and consequently the embryonic development of *K. scorpioides*, is one of the longest for freshwater turtles, as observed in this study. An average duration of 129.50 $\pm$ 21.73 days was reported in the literature for the incubation period [24]. Long incubation periods are well known for the species, with 266 days as the longest period reported [36]. Moreover, our results showed the positive correlation between egg and hatchling mass, this fact is already well established for birds and widely used in poultry farming to separate egg sizes [37,38].

A supposedly initial diapause in an embryo was observed in this work since they were not developed until 40 days of incubation. Even though, this aspect was observed in Kinosternidae [39], the other embryos had a normal development. These findings suggests that the egg incubation management conditions in the present study contributed positively to the development of the embryos.

In this regard, these pauses may occur before hatching while the egg is in the oviduct (pre-ovipositional period), during incubation (post-ovipositional period, embryonic diapause, and cold torpor), and in the late stages of embryonic development (delayed hatching and aestivation) [40]. In reptiles, various forms of diapause enable embryos to respond to different environmental variations [14,41], while in mammals and birds, this mechanism is limited due to parental care [39]. Therefore, the reason for the supposed diapause observed here would need to be investigated.

4.2. Hatchery process

It should be mentioned that important measures had be considered during this work, such as: 1- the eggs remained in the same position found in the nest, which favors non-rupture of the extra-embryonic membranes, which would otherwise lead to the embryo death [28,29]; 2- identification and removal of contaminated eggs, as the proliferation of microorganisms can harm other eggs [16,42]; 3- temperature and humidity control.

The temperature used in our study (28.5 $\pm$ 0.5°C) effectively promoted embryo development. A similar temperature was also considered ideal for the incubation of Asian turtle eggs [43]. Temperature is the main factor influencing embryonic development in chelonians [44]. It can alter the metabolic rate of the embryo, causing changes in the duration of the stages of development [45]. Furthermore, it can influence the fitness of the hatchlings, as those of larger size are seen in clutches at lower temperatures, which consequently, consume more yolk. Despite this fact, low temperatures lead to longer incubation periods, requiring extra time and care from producers [43,46].

The North region of Brazil presents high annual humidity rates [47]. Therefore, in this study, we chose to use similar humidity to that found in the natural environment (80 %) and in previous studies [24]. High humidity can prevent gas exchange in the egg, leading to embryo death. Moreover, low humidity levels are also harmful because they cause dehydration [45,48]. However, in the Asian turtle species *Mauremys mutica* and *Mauremys reevesii* [27,43] humidity only influenced the duration of the incubation period and the width of the hatchlings carapace, so it is identified as a factor of little concern for egg development. Finally, in the studies above, humidity at 1:1.2 (vermiculite to water ratio) was considered optimal for embryo development [43].

Vermiculite, the substrate used in our study, is commonly used in the incubation of reptile eggs [43,49,50], and was also ideal for *K. scorpioides* embryo development. Unlike other substrates, such as sand or sawdust, vermiculite moderately retains moisture. The particle size of the substrate affects the water environment of the egg, consequently altering the temperature and humidity of the location [44]. Hatchlings with a high number of females was found in a substrate made of sandy material, which raises the temperature; the opposite was observed in clay soils, which retain more moisture, consequently reducing the temperature [44].

5. Conclusions

The results of this study demonstrated that candling technique allowed monitoring of the development and quality of *K. scorpioides* embryos. It is a simple and useful tool, required in good management practices to increase the production of hatchlings. Due to the low cost of this method, it can be used for breeders of this species also by conservation researchers.

CRediT authorship contribution statement

Diva Araújo Guimarães: Writing – review & editing, Writing – original draft, Funding acquisition. **José Ribamar Felipe Marques:** Resources. **Maria Auxiliadora Ferreira:** Writing – review & editing, Resources. **Verônica Oliveira-Bahia:** Writing – review & editing, Supervision. **Cassiane Leal:** Methodology. **Brenda Braga:** Writing – review & editing, Writing – original draft, Methodology, Formal analysis, Data curation, Conceptualization.

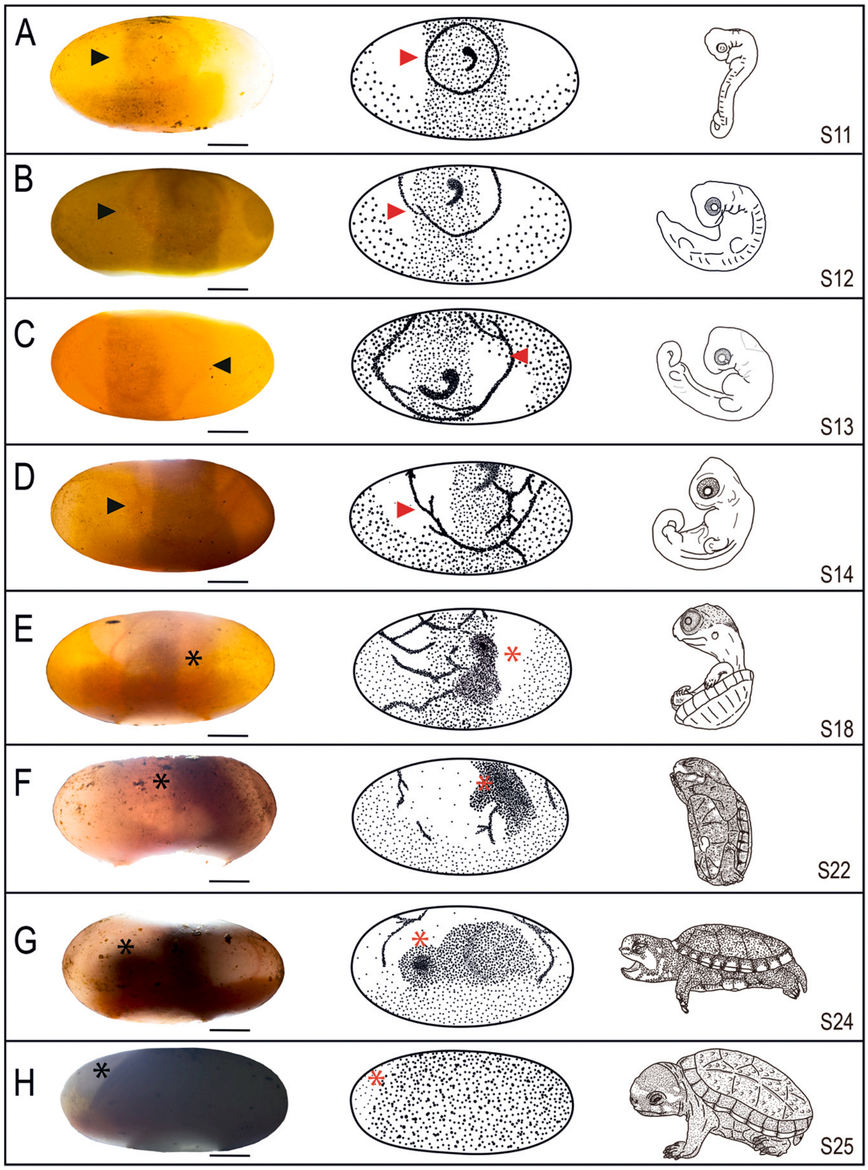

Fig. 4. Candling of fertile *Kinosternon scorpioides* eggs. **Early stages**: A) Formation of the vitelline circulatory system (head arrow) (15 days); B) Development of the vitelline circulatory (20 days). C) Expansion of the yolk circulatory system and embryo lateral decubitus position (28 days); D) Descent of the embryo into the yolk (35 days). **Middle Stages**: E) Embryo resting on the yolk (*) (42 days). F) embryo at the end of this period and rests on the yolk, changing its body position (*) (61 days). **Late stages:** G) increased pigmentation of the embryo, which is seen in the prone position (80 days); H) Difficult to see the inside using candling, only the air chamber can be seen. (*) (95 days). S: embryonic development stages. Scale bar: 1 cm.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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