Ageing the eggs and embryos of the pig-nosed turtle, *Carettochelys insculpta* (Chelonia: Carettochelydidae), from northern Australia

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Abstract: Standard series of embryonic stages are the primary basis for organising information in embryological studies and for ageing eggs and embryos in field studies. In this paper we calibrate the developmental series for the pig-nosed turtle, *Carettochelys insculpta*, from northern Australia against an established series for *Chelydra serpentina*, carefully noting unique attributes of *C. insculpta*. We also extend existing non-destructive approaches to staging embryos by identifying several additional specific embryological attributes visible externally or by candling. A chronological sequence of attributes visible by candling is established as a viable alternative to the destructive approaches requiring direct examination of embryos.

Résumé : Des séries-étalons de stades embryonnaires constituent la base de l'organisation de l'information en embryologie et elles sont essentielles à la détermination de l'âge des oeufs et des embryons au cours d'études sur le terrain. Nous avons calibré une série de stades de développement de *Carettochelys insculpta*, une tortue du nord de l'Australie, en les confrontant à une série déjà établie de stades de *Chelydra serpentina*, notant les particularités de *C. insculpta*. Nous apportons également des détails supplémentaires au sujet des approches non destructrices déjà existantes de détermination des stades embryonnaires en identifiant plusieurs caractéristiques embryonnaires spécifiques additionnelles par observation externe ou par mirage. L'établissement d'une séquence chronologique des attributs visibles par mirage est une alternative intéressante aux approches destructrices qui supposent l'examen direct des embryons.

[Traduit par la Rédaction]

Introduction

A standard series of embryonic stages is the primary basis for organising information in embryological studies (Yntema 1968; Miller 1985*a*). It provides consistency in staging across studies, a basis for comparing development of embryos incubated under differing conditions, and a basis for estimating duration of incubation and predicting hatching date in the field. Techniques for staging embryos or otherwise determining the age of nests under field conditions are also particularly important for species with temperature-dependent sex determination. The thermosensitive period for sex determination (when sex is irreversibly influenced by temperature) occurs in a series of stages, usually in the middle third to half of development (Yntema 1979; Bull and Vogt 1981; Webb et al. 1987). Accurate delimitation of this period, so that the temperatures which influence offspring sex in natural nests can be identified, requires accurate staging of embryos. Many species of freshwater turtle lay moderate-sized clutches (10–25 eggs), so non-destructive techniques for determining this period are particularly valuable.

Stage of development is defined by documenting the progressive appearance of discrete morphological characters. Standard embryological series have been published for turtles (Yntema 1968; Mahmoud et al. 1973; Miller 1985*b*; Renous et al. 1989; Billett et al. 1992; Guyot et al. 1994), crocodilians (Ferguson 1985), lizards (Dufaure and Hubert 1961; Hubert 1985), snakes (Raynaud 1961; Hubert and Dufaure 1968; Hubert 1985), and the tuatara (Moffat 1985). Other studies have drawn upon these standard series to develop less comprehensive series useful in ageing eggs and embryos in laboratory and field studies (Zehr 1962; Magnusson and Taylor 1980; Webb et al. 1983*a*, 1983*b*). Few of these studies include quantitative measurements in conjunction with character data in defining stages (but see Magnusson and Taylor 1980; Webb et al. 1983*a*, 1983*b*; Ferguson 1985). Still fewer involve the use of characters that can be examined non-destructively despite their potential value in staging embryos and ageing nests of threatened species (Ewert 1985).

Ewert (1985) first demonstrated the value of candling reptile eggs, where a bright light is shone through the egg from various angles to reveal internal features. He established a chronology of developmental stages for eggs of 37 species of turtle from six families incubated over a range of temperatures. Features useful in staging by candling include the formation and expansion of the extra-embryonic vitelline circulation and the allantoic sac, and various embryonic attributes. Ewert (1985) identified five stages (corresponding to the standard series of Yntema 1968) based on the sequential appearance of blood islands (stage 5), development of the vitelline circulatory system (referred to hereinafter as the haemodisc) (from stage 8; Fig. 16 in Ewert 1985), eye pig-

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mentation (stage 12), body pigmentation (stage 20), and time of hatching (stage 26). No other studies have made extensive use of candling characters to systematically age embryos or correlate such characters with standard developmental series.

The development of the opaque patch is also useful for ageing in some species. Opaque patches are indicative of normal development because they do not form on infertile eggs or eggs that die very early in development (Blanck and Sawyer 1981; Webb et al. 1983*a*, 1983*b*; Ewert 1985; Thompson 1985). The opaque patch forms as the young developing embryo withdraws water from the pores of the eggshell to facilitate gas exchange; as incubation proceeds, the opaque band or patch increases in size in concert with the expansion of the chorioallantoic membrane (Deeming and Thompson 1991). Spread of the opaque patch has been correlated with age in several studies (Webb et al. 1983*a*, 1983*b*, 1986; Thompson 1985).

The pig-nosed turtle, *Carettochelys insculpta*, is a species from the wet–dry tropics of northern Australia that exhibits temperature-dependent sex determination (Webb et al. 1986). Only male offspring are produced at constant temperatures below 32°C and only females are produced at constant temperatures above 32°C (A. Georges, unpublished data). The thermosensitive period for *C. insculpta* has been determined to fall within the middle third of incubation (J. Young and P. West, unpublished data). Each year during the dry season, females crawl out onto sandbanks adjacent to water to deposit a clutch of between 4 and 19 eggs in a shallow chamber (Georges and Kennett 1989). The eggs are white, hard-shelled, and spherical. The eggs incubate rapidly, for 50–90 d depending on incubation temperature, until the embryos reach maturity. They subsequently hatch in response to torrential rain or inundation (Georges and Rose 1993) or enter aestivation within the egg until hatching is triggered (Webb et al. 1986). Immediately prior to aestivation or hatching, a substantial residual yolk body is internalised.

In this paper we establish a developmental series for *C. insculpta*. The primary objective of the study was to develop criteria for ageing the eggs and embryos of *C. insculpta*, not to produce a detailed standard embryological series (sensu Yntema 1968; Miller 1985*b*). We first use the traditional approach of directly examining embryos dissected from eggs incubated at a constant temperature and relating the observations to a standard turtle-embryology series (Yntema 1968). The approach was essentially to calibrate the developmental series for *C. insculpta* against that of *Chelydra serpentina*, carefully noting unique attributes of *C. insculpta*. Second, we extend Ewert's (1985) non- destructive approaches to staging embryos by identifying several additional specific embryological attributes visible externally or by candling.

Materials and methods

Eggs of *C. insculpta* were collected from the Daly River, Northern Territory. Sandbanks adjacent to the water were inspected regularly for evidence of nesting (distinctive tracks and depressions in the sand) during the 1997 nesting season (late July to early October). The sand was probed with 2-mm spring-steel rods to locate nests (after Blake 1974), and nests were regarded as fresh if they were associated with fresh tracks and diggings. The top of each egg was marked with pencil as it was uncovered so that its orientation could be maintained during transport to avoid movement-induced mortality (Limpus et al. 1979; Parmenter 1980). Eggs were buried in moist sand or vermiculite in insulated boxes and transported by boat to Oolloo Crossing (a distance of 20–70 km, depending on site of collection), then by a well-maintained unsealed road (41 km) to Douglas-Daly Research Farm (Australian Department of Primary Industry and Fisheries). Temperature was not directly measured during transport; however, cool, moist sand, insulated containers, and continual shading were used to maintain temperatures below 24°C.

Maximum and minimum egg diameters were measured with vernier calipers (±0.1 mm) and egg mass was determined with an electronic field balance (±0.1 g). Egg viability was assessed by checking for the development of the opaque patch (Thompson 1985) within 24 h of egg laying. Each egg was candled by shining a fibre-optic light source (120 W) from various angles to reveal any gross embryonic structure. Eggs were then buried in moist vermiculite (four parts water to three parts vermiculite by mass) in plastic containers with each egg occupying its own compartment. Small holes were made in the lids to allow gases to diffuse while minimising water loss. The containers, with 18 eggs in each, were weighed to monitor water loss, and water was replenished when required. The containers were placed in constant-temperature incubators (Thermoline Model RI 170, refrigerated). Water trays were placed in the bottom of each incubator to maintain high but unmeasured humidity. The apparatus was monitored at hourly intervals before the eggs were added, to ensure that temperatures were held constant within acceptable limits (±0.5°C). Once eggs were placed in the incubators, temperatures in close proximity to the eggs were monitored daily throughout incubation using a mercury bulb thermometer (±0.1°C) calibrated against a thermometer certified as accurate by the National Authority of Testing Agencies. Every second day, the temperature was checked on an hourly basis over an 8-h period to confirm that incubation temperatures were operationally constant. Temperatures remained within 0.5°C of the nominated temperature throughout incubation.

One clutch of 9 eggs (CAI97_140), for which the laying date was known, was selected to establish a detailed embryological series for calibration against Yntema's (1968) standard series. It was laid on 18 August 1997, collected on 19 August 1997 and allocated to a 30°C constant incubator on 21 August 1997. A temperature of 30°C produces only males. One egg from this clutch was opened after each of 32, 34, 38, 44, 48, 54, 60, 70, and 78 d incubation, corresponding to stages 18–26. The correspondence between age and stage was initially estimated from the data of Webb et al. (1986). A further 41 eggs from 14 clutches were incubated at 30°C as part of other experiments not reported here. Their exact date of laying was not always known, but they were selected because candling revealed no indication of blood islands (before stage 5) or other features indicating that development was well advanced. The starting point for estimating age was taken as the day a haemodisc first appeared, and an estimate of the laying date at 30°C was obtained later using criteria established from the developmental series for clutch CAI97_140 above. Embryos from these clutches were used as specimens for stages 12–17 of the embryological series and to provide additional candling opportunities. One further clutch was obtained by hormonal induction using oxytocin (Ewert and Legler 1978) and 4 eggs were placed in incubators set at 30 and 34°C (2 eggs each) to monitor the development of the opaque patch. The eggs described above were candled every second day, with the exception of the eggs used for measuring the opaque patch, which were initially candled every 2 h for the first 72 h.

Early-stage embryos were killed by chilling the eggs to below 5°C for a minimum of 36 h. Late-stage embryos were killed by intracranial injection of pentabarbitone. They were separated from the yolk and extra-embryonic membranes, blotted dry, and weighed (±0.1 g). They were then examined under a stereomicroscope,

tentatively staged against Yntema's (1968) standard series while fresh, then fixed in 10% buffered formalin. Maximum head width (including the optic capsules) was measured on small embryos by means of a calibrated eyepiece (±0.1 mm) and on larger embryos by means of vernier calipers (±0.1 mm). The head-width ratio (HWR) is expressed as the ratio of head width to egg diameter. Detailed staging of embryos was undertaken after fixation. Particular attention was paid to features unique to *C. insculpta*. Where possible, multiple specimens at the same stage were compared to establish variability in the relative development of different characteristics at each stage. Specimens typical of each stage were selected for photography. In some cases the embryo may have been slightly more or slightly less developed than the exact stage. This is indicated on the figures by a plus or a minus sign.

Measurements recorded during external examination and candling of eggs included (when visible) the following: maximum and minimum diameter of the opaque patch, diameter of the haemodisc parallel to and perpendicular to the long axis of the embryo, width of the allantoic sac in dorsal view, maximum embryo crown–rump distance as projected against the eggshell, maximum projected embryo carapace length in lateral view, and embryo orientation. Crown–rump distance was measured in dorsal view for stages where a carapace was not distinguishable and in lateral view for stages where a carapace was visible. All measurements were taken with calipers as straight-line measurements and hence did not take into account curvature of the shell. These measurements were converted to indices as follows. The opaque-patch index (OPI) and haemodisc index (HI) are each expressed as the average of their two measurements (length and width) divided by the average egg diameter. The allantoic-sac index (AI), crown–rump index (CRI), and carapace-length index (CLI) were each calculated by dividing the length by the average egg diameter.

Values are given as the mean with standard error, unless otherwise specified.

Results

Direct embryo measurements

Detailed definitions of the stages of development of *C. insculpta*, based on the standard series for *C. serpentina* (Yntema 1968), are given in Table 1 and illustrated in Figs. 1–9. *Carettochelys insculpta* embryos at stages 12–17 differed only in minor respects from the standard series. Differences involved pigmentation (grey rather than black) and the timing of its appearance. Beyond stage 17, features unique to *C. insculpta* became apparent. The two digits that would ultimately bear claws were differentiated from the remaining digits from stage 18, and the two claws were a distinguishing feature from stage 20. The flipperlike limb structure of *C. insculpta* was first evident from stage 21. The distinctive fleshy proboscis and the white patch posterior the eye were evident from stages 21–22. The tuberculate median keel of *C. insculpta* was evident from stage 21. The scute pattern described for the standard series of *C. serpentina* (from stage 15) was never evident, alternating ridges and depressions of the rib arches being the distinctive carapace feature in *C. insculpta*. Eyelid development in *C. insculpta* lagged behind that of *C. serpentina* compared with the development of other features useful in diagnosing embryonic stages.

HWR increased linearly with time up to stage 24 (ca. 60 d), reaching a maximum of 0.33 ($F_{[1,15]} = 150.5$, $p < 0.001$). This linear relationship is described by the equation

[1] $\text{HWR} = 0.0048 \text{ age} + 0.0148$ ($r^2 = 0.91$)

where age is measured in days. Beyond stage 24, up to the point of hatching (stage 26), HWR remained constant.

Stage increased linearly with HWR up to stage 24 (HWR = 0.33), a relationship used only to estimate stage from HWR because stage is an ordinal-level measurement. For descriptive purposes only, this relationship is described by the equation

[2] $\text{Stage} = 55.94 \text{ HWR} + 8.37$ ($r^2 = 0.95$)

Similarly, the third relationship, between stage and age (up to and including stage 24), is given by

[3] $\text{Stage} = 0.30 \text{ age} + 7.87$ ($r^2 = 0.89$)

If prediction is required up to stage 26 (hatching), then the log-linear relationship

[4] $\text{Stage} = 19.28 \log_{10} \text{age} - 10.46$ ($r^2 = 0.97$)

is a satisfactory predictor.

Candling observations

Many consistent features were visible during candling and several were associated with specific embryonic stages (Table 2). Careful tilting of eggs during candling did not harm embryonic development. The opaque patch was the first characteristic observed, appearing about 18 h after laying. After 7 or 8 d, blood islands developed. These anastomosed into a well-defined haemodisc, which was first visible at stage 8 (days 8–10). The outline of the allantoic sac was visible by stage 13 (days 15–18). The embryo itself was visible from about day 10, and the limbs and carapace were distinguishable at stages 14 (days 18–20) and 17 (days 25–29), respectively. Eye pigmentation was evident from stage 12 (days 14–15), though it was not clearly visible in many cases. Embryonic pigmentation was detectable caudally at stage 20 (days 36–41). Limb movement was consistently detectable from stage 19 (days 32–36), and coordinated "swimming" actions were evident from stage 21 (days 41–46). Late in development, from stage 23 (days 52–59), no specific embryonic characteristics were visible and overall size and evidence of yolk were the only features that could be consistently described (Table 2).

Embryo orientation changed consistently at various stages of development. Early in development, the embryo was oriented so that its left lateral face was presented to the yolk. Body position was parallel to the vertical axis, with the posterior end oriented towards the dorsal pole (position 1; Fig. 10A). The embryo was found in this position from its initial distinction at stage 8 until stage 14, when it began to rotate with the expansion of the allantoic sac (Fig. 10B). In the second orientation (stage 16), the embryo had rotated to a position perpendicular to its initial position (position 2; Fig. 10C). By stage 18, the embryo had rotated inwards, so that the ventral surface was facing the vertical axis (position 3; Fig. 10D). At stage 19, the embryo was in the process of rolling so that the ventral surface faced the yolk. The best view of this position was obtained by gently rotating the egg from left to right and slightly tilting its dorsal pole towards the observer. From day 36 (stage 20), the embryo rotated to present its ventral face to the yolk (position 4; Fig. 10F). At

Table 1. Descriptions and measurements of stages 12–26 in the development of *Carettochelys insculpta,* based on embryos incubated

Stage	Age (days)	Embryo HWR	Cranial features	Limbs
12	14–15	0.0820–0.0868	**Pharyngeal slits are not visible. Maxillary process extends as far ventrally as the mandibular. Retina is pigmented** and light grey	Limb buds are longer than they are wide, but hind limb buds are only slightly longer than wide. Limbs point more caudally than ventrally
13	15–18	0.0868–0.1012	**Maxillary process extends beyond the mandibular and limits a well-marked nasolacrimal groove posteriorly**	Both fore- and hind-limb buds are clearly longer than they are wide
14	18–20	0.1012–0.1108	**Maxillary and lateral nasal processes are fused. Mandibular process is inconspicuous.** Retina is dark grey	**Limb is an early paddle stage with the digital plate vaguely indicated**
15	20–22	0.1108–0.1204	**Mandibular process extends to the posterior eye level**	**Digital plate is well formed with no digital grooves present**
16	23–25	0.1204–0.1348	**Lower jaw extends to just behind the level of the lens. Scleral papillae are indicated**	**Digital plate has a smooth periphery and slight indications of digital ridges**
17	25–29	0.1348–0.1540	**Lower jaw extends beyond the level of the lens but does not reach the frontal process. Scleral papillae are distinct.** Nasal process is conspicuous	**Periphery of the digital plate is slightly serrated. Digits are indicated by five ridges and four intervening grooves**
18	29–32	0.1540–0.1684	**Lower jaw ends at the frontonasal groove, which is conspicuous.** Free end of the lower jaw is pointed. Eyelid development is visible and upper and lower lids are just touching	Digital plate has clearly indicated digits. The first two digits project beyond the webs for a distance less than their width at the web. Remaining digits do not project beyond the webs
19	32–36	0.1684–0.1876	**Frontonasal groove persists.** Eyelid appears complete around the eye as the upper and lower lids meet, but the width of the lid is less at this point than for the rest of the eyelid. Nostrils can be seen as pale circles at the end of the nasal process	The first two digits project beyond the web for a distance greater than or equal to their width at the web
20	36–41	0.1876–0.2116	Width of the eyelids is uniform. Lower lid reaches the scleral papillae. Nostrils appear as very small depressions	Claws on the first two digits (delimited at the level of the webs) are visible under low magnification. Faint grooves running posteriorly across the anterior edge of the limbs are visible under high magnification
21	41–46	0.2116–0.2356	Lower eyelid reaches the level of the lens and the scleral papillae are no longer conspicuous. A small white patch is vaguely indicated behind the eye	Claws are clearly delimited. Deep grooves on the anterior edge of the limbs are visible under low magnification. Overall, the limbs appear flipperlike
22	46–52	0.2356–0.2644	Lower eyelid reaches the level of the lens. Nasal process appears more like a proboscis and nostrils are visible as small depressions. White patch behind the eye is conspicuous	Claws are enclosed in "sheaths." Grooves on the anterior edge of the limbs are just visible without magnification
23	52–59	0.2644–0.2980	Lower eyelid crosses the lower margin of the lens. White patch behind the eye is very distinct	Grooves on the anterior side of the limbs are clearly visible
24	59–66	0.2980–0.3316	Lower eyelid covers less than half of the pupil. Proboscis and nostrils are well formed. Skin folds are apparent at the base of the neck	As for stage 23
25	66–77	0.3316	Lower lid covers more than half of the pupil, but the eyelids are not able to fully close	As for stage 23
26	≥77	0.3316	Eyelids are fully formed and able to close. Proboscis and nostrils fully formed; the hatchling has a fleshy "pig" nose	Claws are no longer enclosed in sheaths

Note: The range in head width ratio (HWR) for each stage was predicted from age using eq. 1. Low magnification is 6× and high magnification is 16×.

at 30°C.

Carapace	Pigmentation and other features	Figure(s)
		1
		1
A groove on the lateral trunk indicates a demarcation of the carapace. Central and lateral rib bars and intervening bands are vaguely outlined so that when they are viewed laterally, grooves can be seen along the dorsum of the embryo		1
Carapace is clearly limited laterally, but indistinctly so in front and behind	**Part of the intestine is herniated through the body wall** and appears as a "loop"	1
Carapace is clearly limited around its periphery. Central and lateral rib bars and intervening bands are distinct		2 and 7
Marginal rib bars and intervening bands are vaguely indicated		2 and 7
Periphery of carapace is smooth		2 and 7
Periphery of carapace is slightly irregular. Central and lateral rib bars and intervening bands are slightly corrugated	Pigmentation is visible, but pale on forelimbs, carapace, tail, and face. Pigmentation is visible on the nasal process under high magnification only	3 and 7
Periphery of the carapace is serrated. Small tubercules are visible on the median dorsum of the carapace	Pigmentation is visible on all areas except the claws. In particular, the outer edges of the nostrils are lightly pigmented. Pigmentation is paler on the dorsum and marginal areas of the carapace than in other areas	3 and 8
Serrations at the periphery of the carapace are "hooked." A turberculate median keel is visible on the dorsum of the carapace	In general, the embryo is light grey with darker pigmentation on the marginal areas of the carapace and limbs	4 and 8
As for stage 21	Pigmentation is visible in all areas, but pale grey and unpigmented areas persist. Very faint pigmentation is visible at the base of the claws. Pigmentation around the edge of the nostrils is dark grey. In general, the embryo is mid-grey	4 and 8
Posterior edge of the carapace is "bent" ventrally and the plaston is "creased" from being confined in the egg	**The loop of the gut, which has been herniated since stage 15, has been drawn back into the body.** Overall, pigmentation is dark grey, darker than at the previous stage, and conspicuous at the base of the claws	5 and 8
Loose flaps of skin make up the posterior periphery of the carapace	Light-grey pigmentation is evident on the claws, from the base to about two-thirds of their length	5 and 9
As for stage 24	Pigmentation on the claws is dark grey. The yolk is internalised during this stage and may appear to be "halfway" inside the body of the embryo	6 and 9
Posterior edge of the carapace is soft and flexible just after hatching. This straightens and stiffens within the first week after hatching to form a strongly serrated margin	Dorsal surface of the hatchling is dark grey on the carapace, head, and limbs (with some pale-grey areas). On the plastron and ventral surfaces, the hatchling is light grey. The deep crease in the plastron disappears soon after hatching	6 and 9

Boldface type denotes features of *C. insculpta* embryos that are the same as those identified for *Chelydra serpentina* by Yntema (1968).

Fig. 1. Lateral (right) views of *Carettochelys insculpta* embryos at stages 12–15.

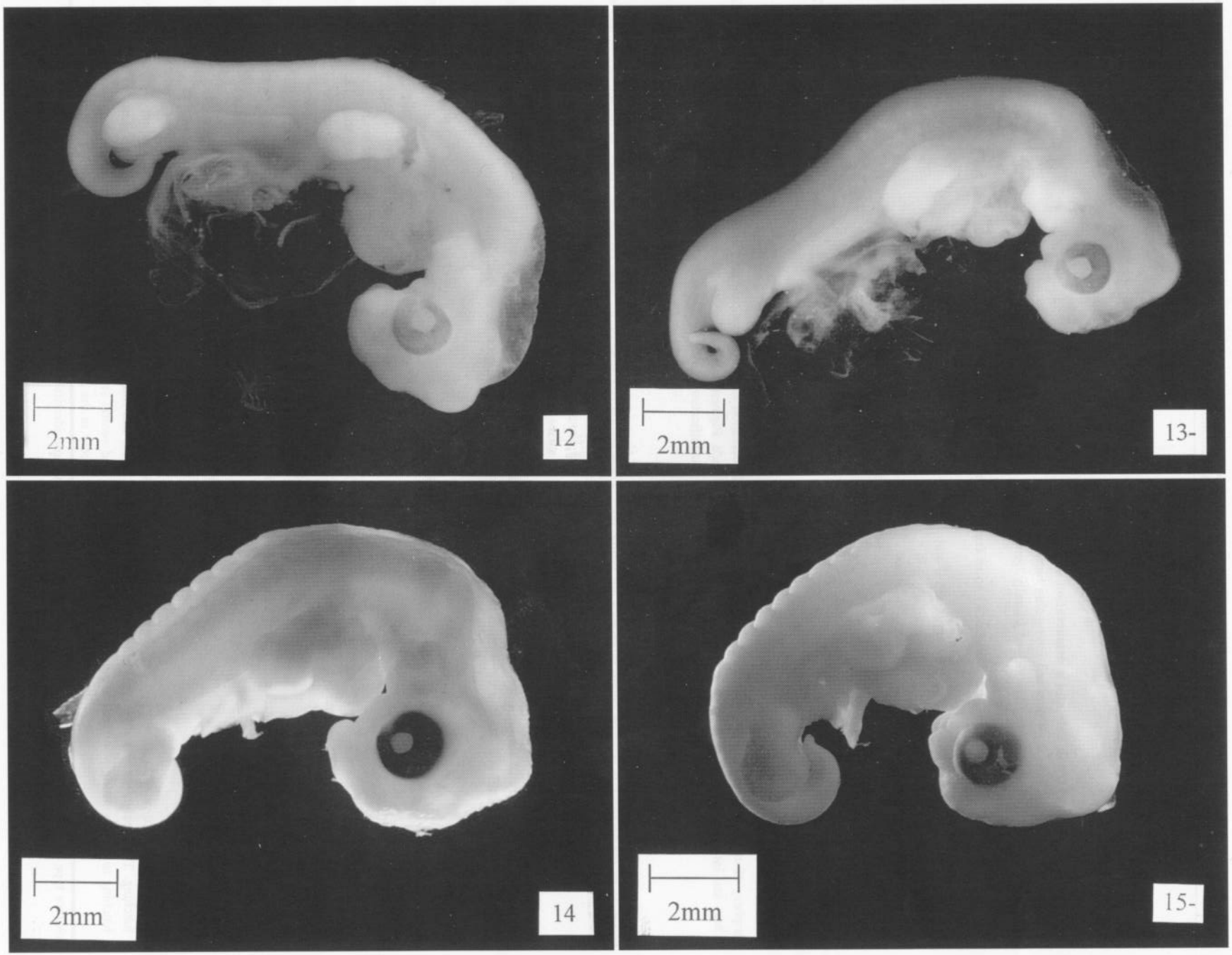


Fig. 2. Lateral (right) view of a whole *C. insculpta* embryo at stages 16–18 and anterior view of the head at stage 18.

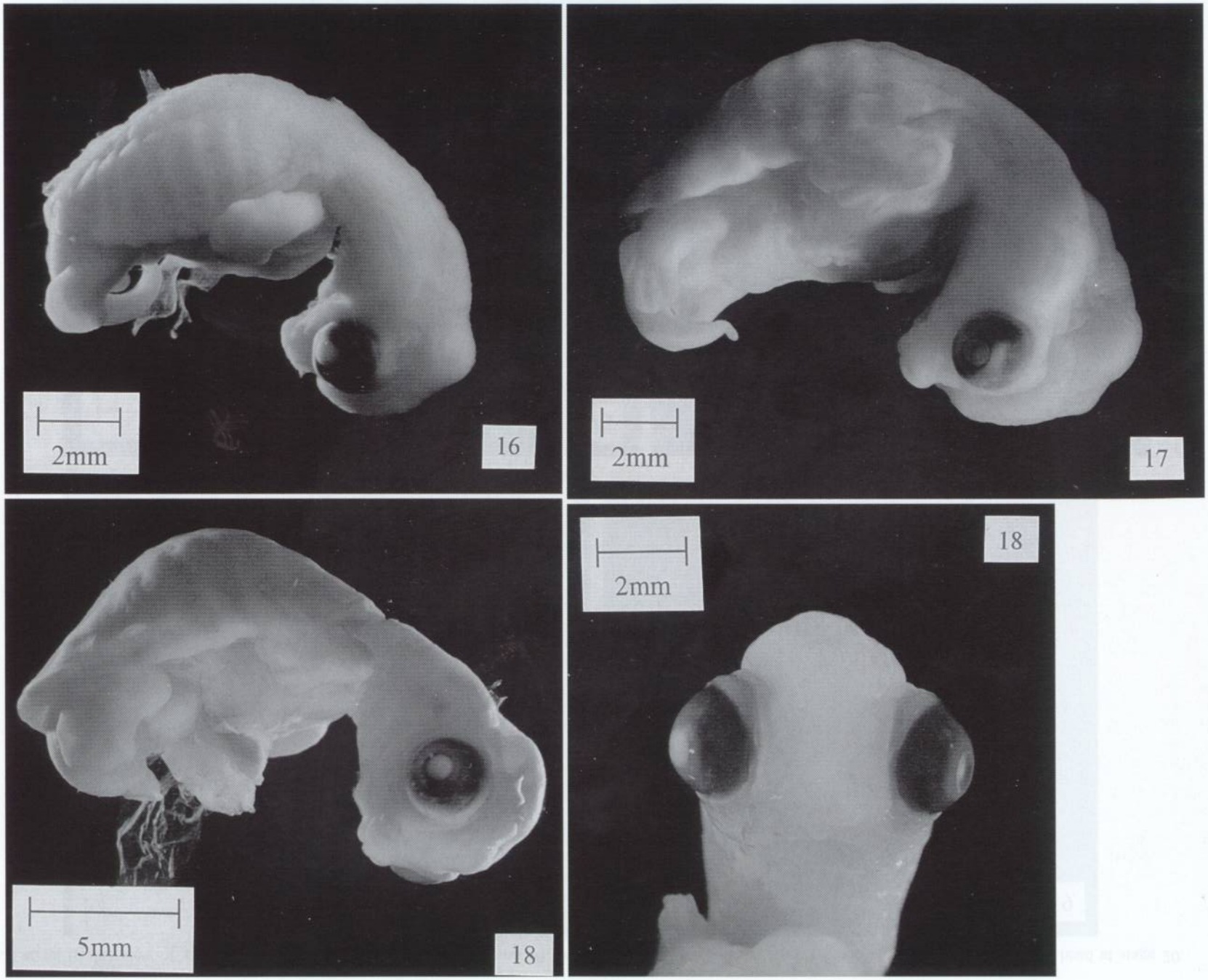


Fig. 3. Lateral (right) view of a whole *C. insculpta* embryo at stages 19 and 20, anterior view of the head at stage 19, and anteroventral view of the head at stage 20.

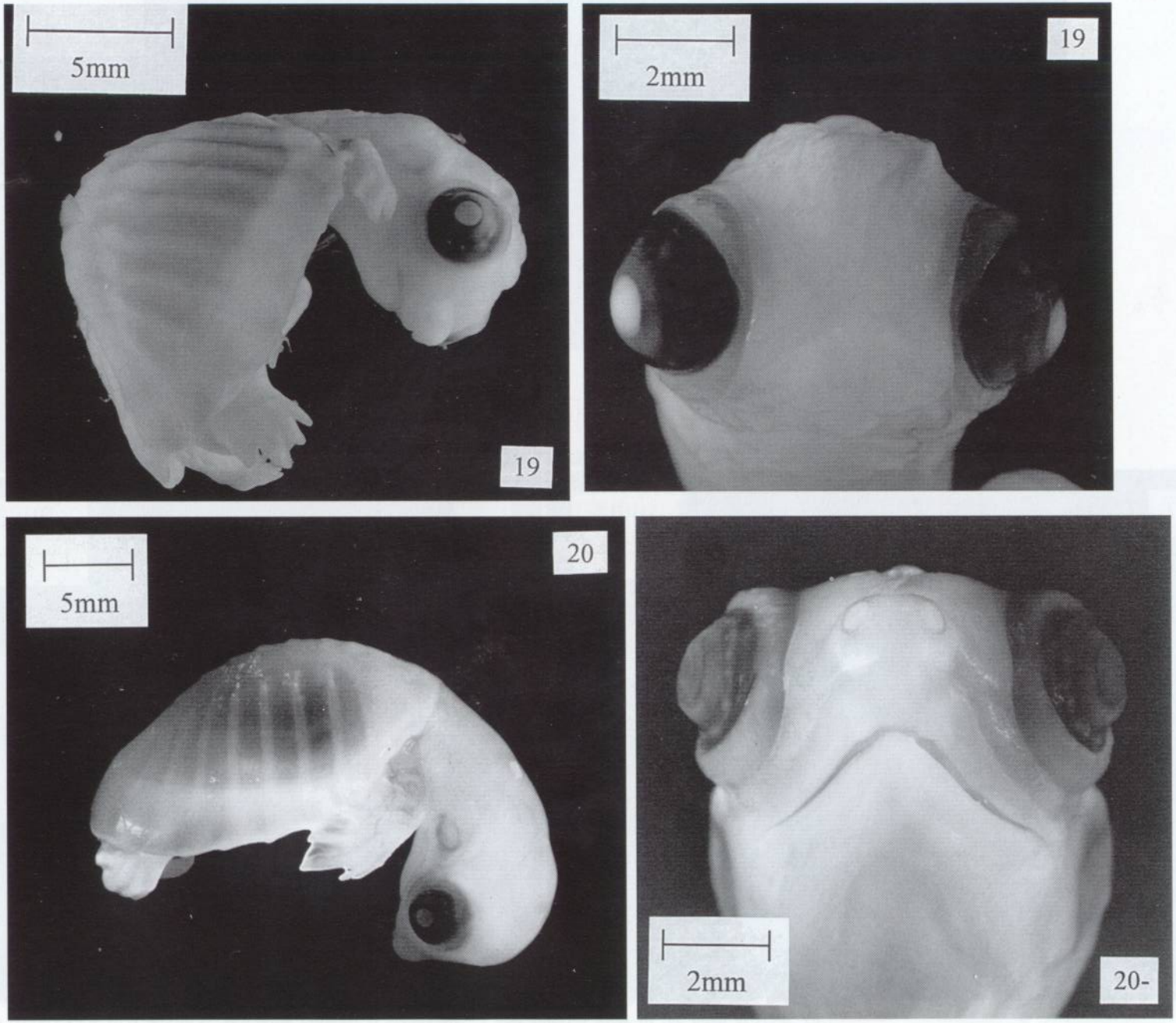


Fig. 4. Dorsal view of a whole *C. insculpta* embryo at stage 21, lateral (right) view of a whole embryo at stage 22, and lateral (right) views of the head at stages 21 and 22.

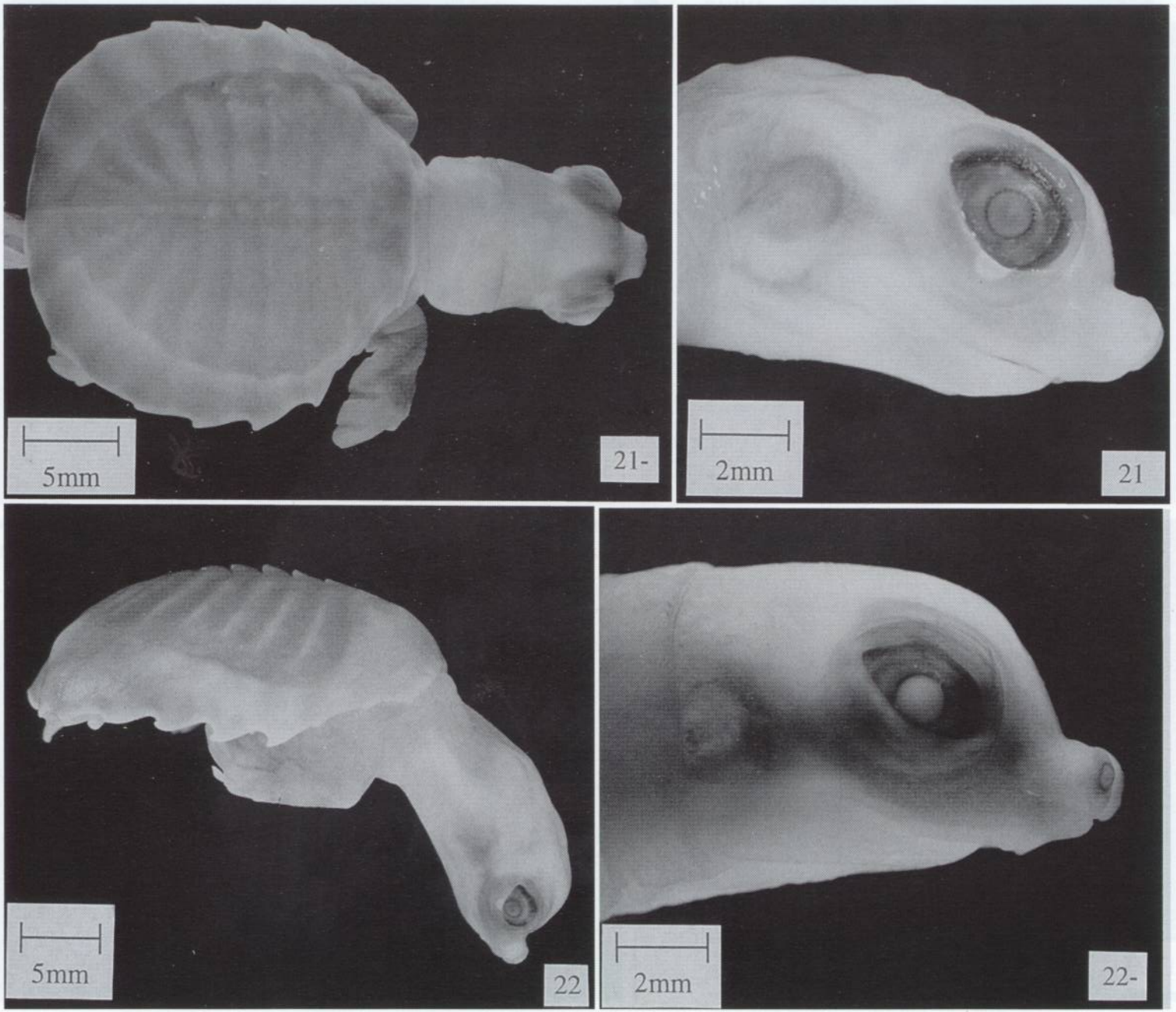


Fig. 5. Lateral (right) views of a whole *C. insculpta* embryo and the head at stages 23 and 24.

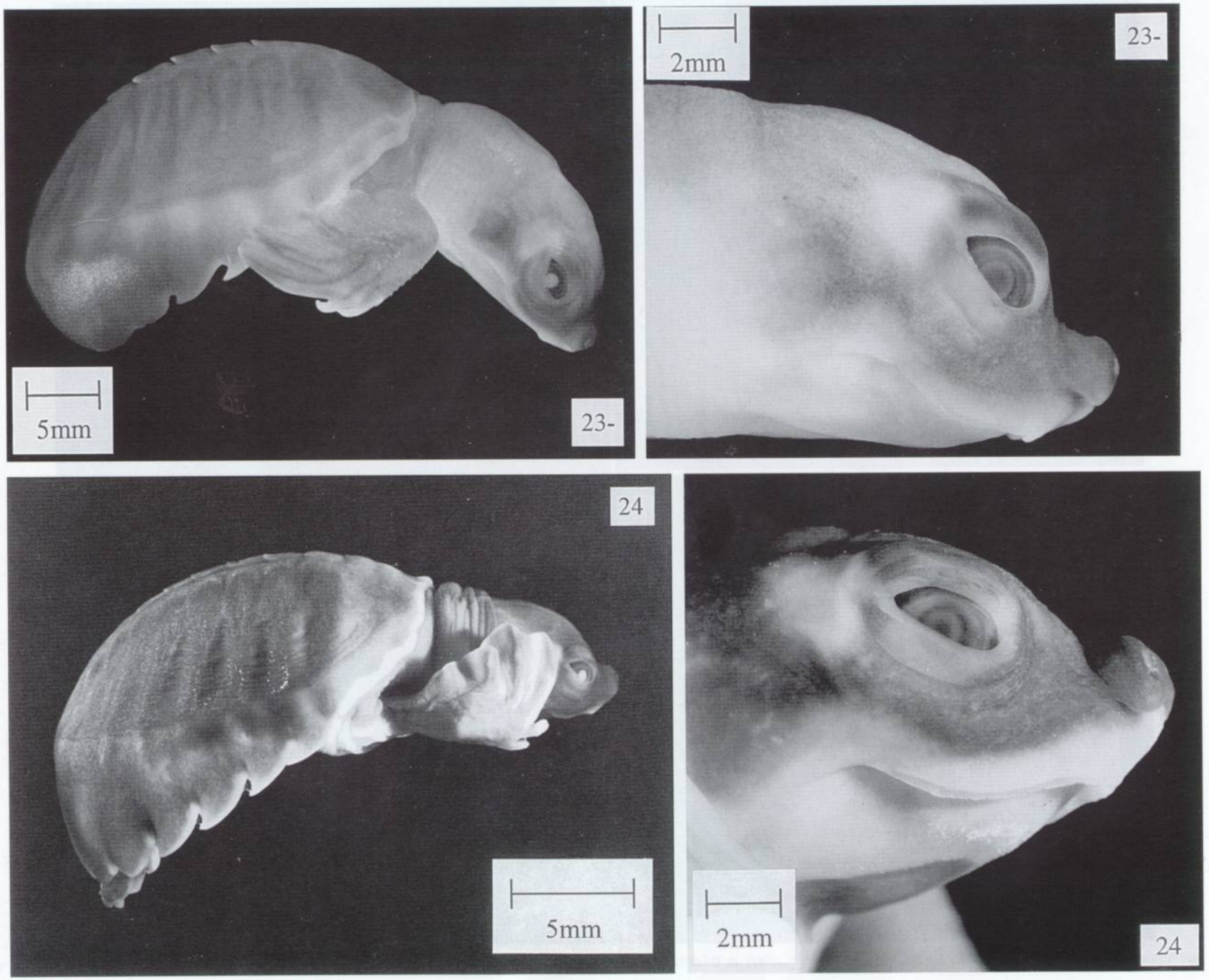


Fig. 6. Lateral (right) views of a whole *C. insculpta* embryo and the head at stages 25 and 26 (hatchling).

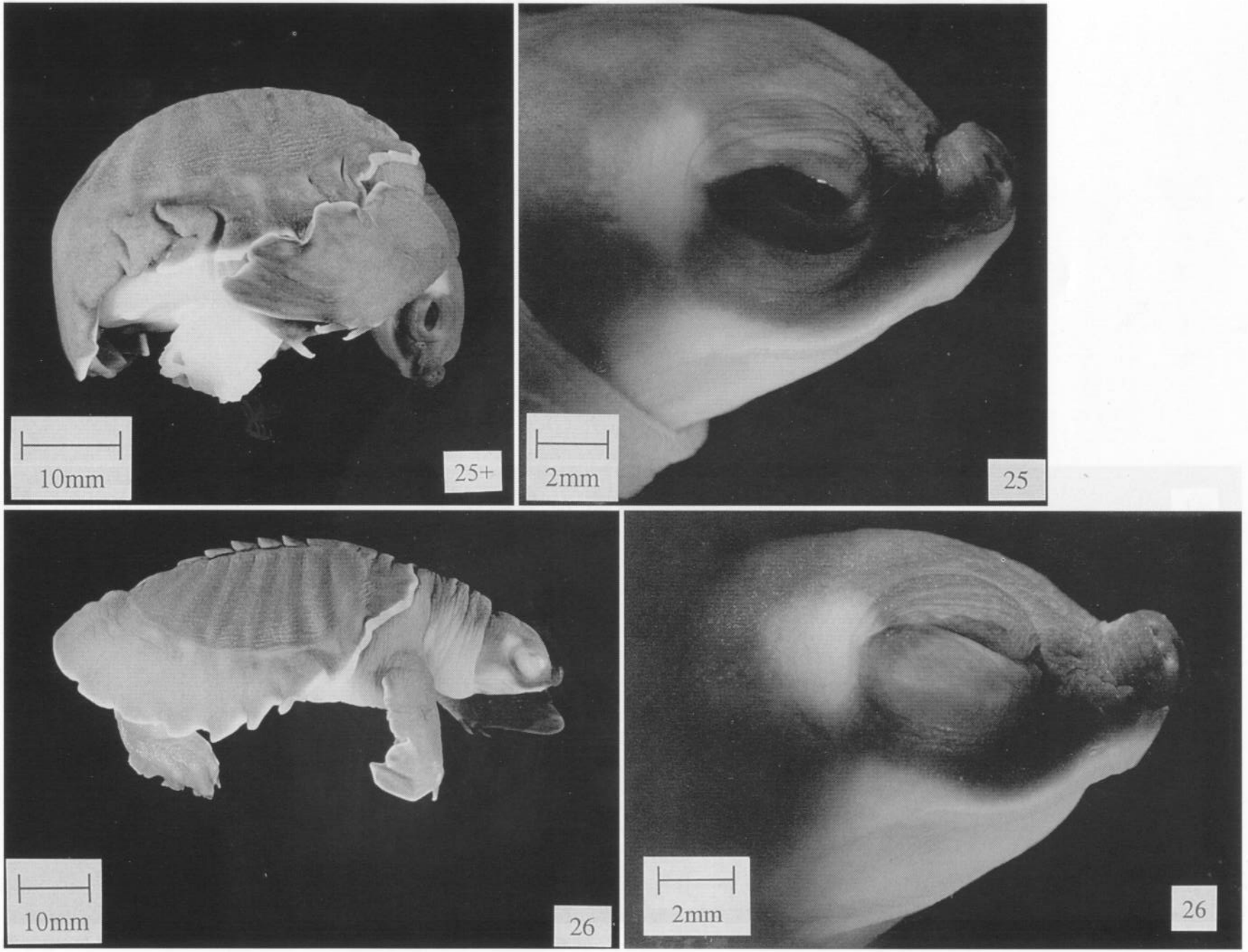


Fig. 7. Dorsal views of the right forelimb of a *C. insculpta* embryo at stages 16–19.

Fig. 8. Anterior view of the right forelimb of a *C. insculpta* embryo at stage 20 and dorsal views of the right forelimb at stages 21–23.

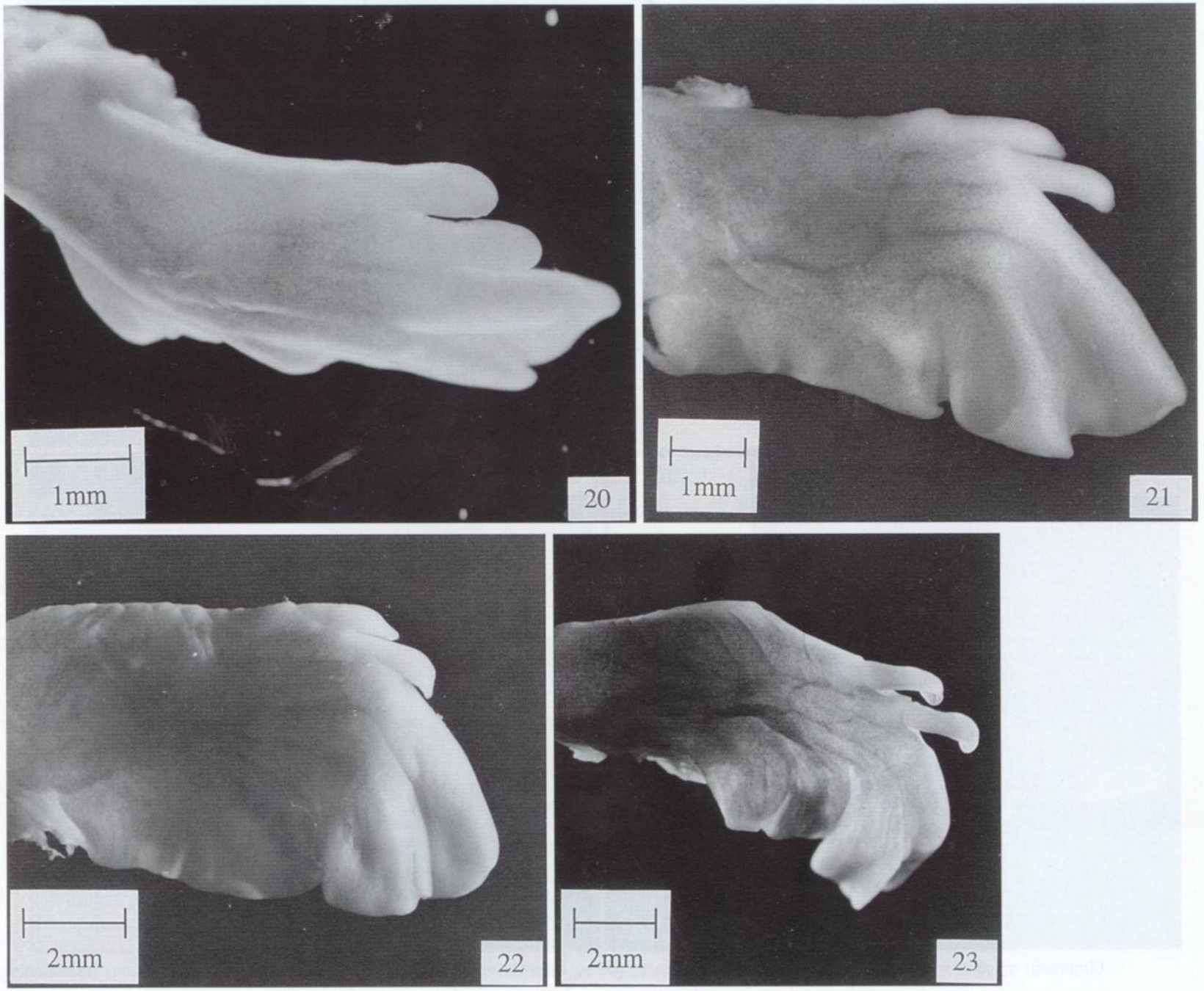


Fig. 9. Anterodorsal views of the right forelimb of a *C. insculpta* embryo at stages 24 and 25 and dorsal view of the right forelimb at stage 26 (hatchling).

Table 2. Qualitative features of normal development of *C. insculpta* embryos, based on candling eggs incubated at 30°C.

Stage	Age (days)	Notes on candling
—	<7	Horizontal layers are visible when viewed laterally (embryonic fluids at the top and yolk at the bottom of eggs). A small opaque white patch is visible on the dorsal surface of the egg (Table 3)
5–6*	7–8	A blood island is visible as a small pale pink spot that forms within the embryonic fluids between 5° and 45° below the dorsal pole of the vertical axis
7–8*	8–10	Blood islands have anastomosed and now appear as a measurable haemodisc. Embryo appears as a partial "cleavage" (straight) line running radially across the haemodisc
9–11*	10–14	Embryo appears curved and thicker laterally as development progresses. Embryo moves independently of the haemodisc when the egg is "jiggled"
12	14–15	Head of the embryo is distinguishable, hence the anterior and posterior ends of the embryo can be differentiated and crown–rump measurement is possible. Eye pigmentation may be visible. Embryo is in position 1 (Fig. 10A)
13	15–18	Outline of the allantois is visible and its width is measurable
14	18–20	Limb buds are distinguishable and the haemodisc is as wide as the egg diameter. Posterior end of the embryo follows the spreading of the allantois. Embryo is between positions 1 and 2 (Fig. 10B)
15	20–22	Embryo orientation continues to follow the spreading allantoic sac; embryo appears in a position between those shown in Figs. 10B and 10C
16	22–25	Embryo has completely moved into position 2 (Fig. 10C), which is perpendicular to position 1 (Fig. 10A)
17	25–29	Embryo carapace is distinguishable and its length measurable
18	29–32	Embryo moves into position 3 (Fig. 10D), with the ventral surface facing the vertical axis. Dorsal anterior and posterior edges of the carapace are clearly distinguishable
19	32–36	Limbs are moving and the width of the allantoic sac is about equal to the egg diameter. Embryo is between positions 2 and 3 (Fig. 10E)
20	36–41	Embryo orientation has changed: the ventral side is facing the yolk and the neck is extended and arched downwards (position 4; Fig. 10F). Tail is visible and appears pigmented
21	41–46	A swimming action is observable and hind-limb pigmentation is evident. Embryo is in position 4 and now exhibits controlled head movements (Fig. 10E)
22	46–52	Early in this stage, forelimb pigmentation is evident. Late in this stage, most of the embryo appears pigmented and crown–rump distance exceeds egg diameter and can no longer be measured reliably
23	52–59	Embryo occupies approximately or just less than half the volume of the egg
24	59–66	Embryo occupies 50–70% of the egg volume
25	66–77	Embryo occupies about 70–80% of the egg volume and the rest of the space remains yellowish (yolk is not fully internalised)
26	≥77	Embryo occupies more than 80% of the egg volume and the remaining space appears whitish (yolk is fully internalised). Embryo is full-term

Note: Stage was verified by opening the eggs after they were candled and examining the embryos.
*No embryos were killed at these stages, therefore those shown are only estimates.

this time the neck was still extended and arched downwards. By stage 21, the embryo was able to retract its neck and the turtle now appeared to be looking outwards (Fig. 10G). The views presented in Fig. 10 are indicative of the clearest view, obtained by rotating the egg in the horizontal plane in front of the light; therefore any rotation of the embryo in the horizontal plane with respect to its eggshell was not recorded.

In general, there were strong predictive relationships between developmental indices derived from candling measurements and age: all indices increased monotonically with age, except CRI, which initially declined (Fig. 11A), presumably as a result of changing embryo curvature with time. In most cases, coefficients of determination were sufficient to support good prediction: CRI, $r^2 = 0.97$; CLI, $r^2 = 0.97$; HI, $r^2 = 0.99$ (Fig. 11). The exception was AI, which showed a relatively poor relationship to age ($r^2 = 0.75$).

The opaque patch increased linearly with age up to about 60 h, then remained approximately constant at about 0.95× egg diameter through much of incubation. For the first 60 h, the relationship between patch diameter and age is given by

$$[5] \quad \text{OPI} = 0.0195 \text{ age} - 0.1923 \quad (r^2 = 0.95)$$

Only towards the end of incubation (beyond stage 24) during the laboratory experiments did the patch ultimately expand to encompass the whole egg. Field observations indicated that the timing and rate of spread of the opaque patch beyond the diameter of the egg is highly unpredictable.

Table 3 shows the relationship between the various quantitative candling attributes and age/stage for *C. insculpta*, and the range of ages/stages to which each attribute is applicable. It is evident that combinations of qualitative and quantitative observations are required for adequate staging of intact eggs. If qualitative candling observations alone were used to age embryos, stage or age could often only be determined as a range (e.g., between stages 14 and 16 or 17 and 18). Using quantitative measurements alone has similar limitations. The CRI, a straight-line measurement, decreased

Fig. 10. Drawings of *C. insculpta* embryo orientation relative to development of the haemodisc and allantoic sac, as seen during candling. The dotted vertical lines represent the vertical axis. Embryos are shown in position 1 (A), between positions 1 and 2 (B), in position 2 (C), in position 3 (D), between positions 3 and 4 (E), and in position 4 (F and G). The drawings are intended to depict gross embryo orientation rather than morphological detail. Slight and gentle rotation of the egg during candling enables the observer to achieve the clearest view. Note that the rotation of the embryo in the lateral plane with respect to the eggshell was not recorded and is therefore not reflected in these diagrams.

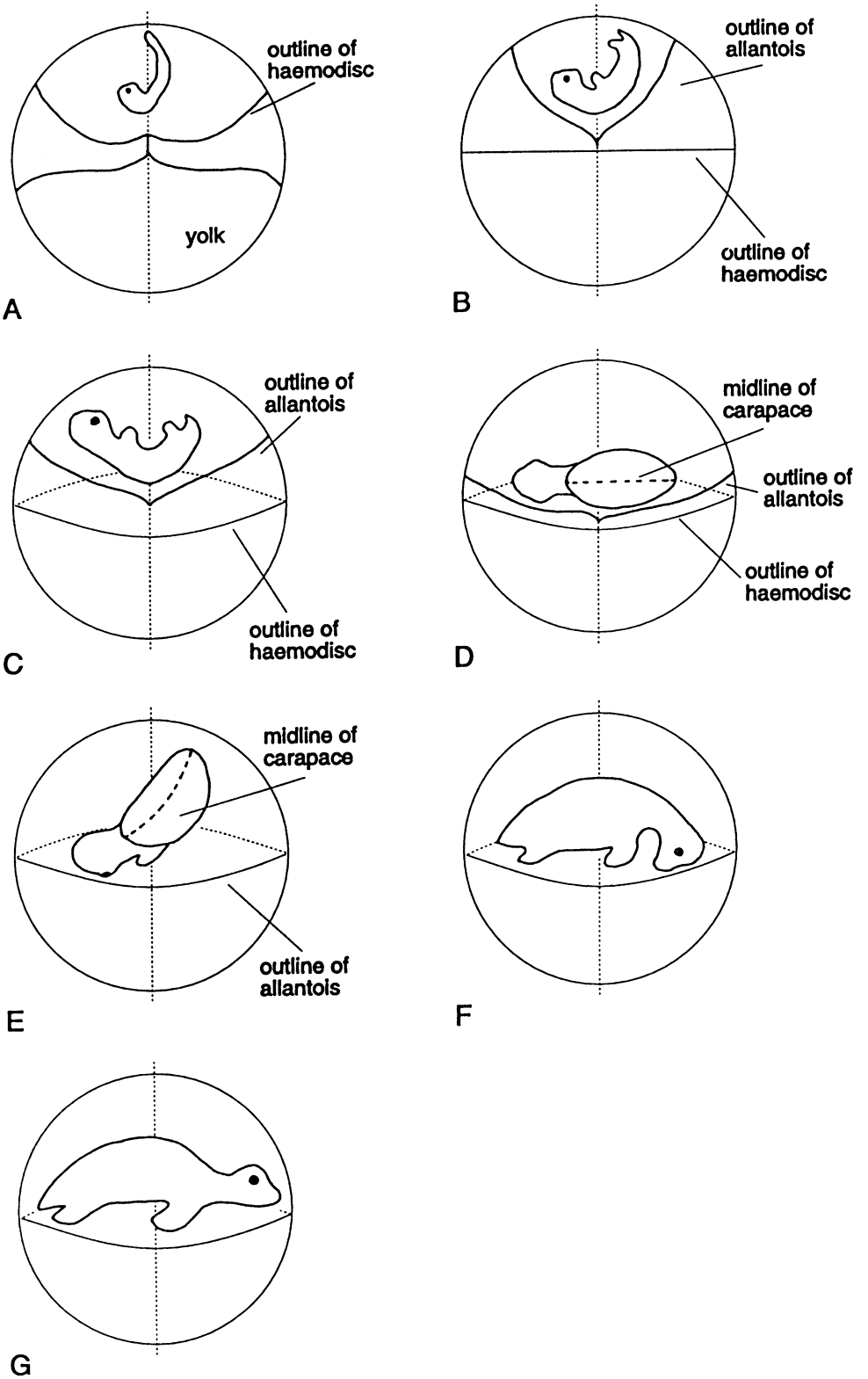
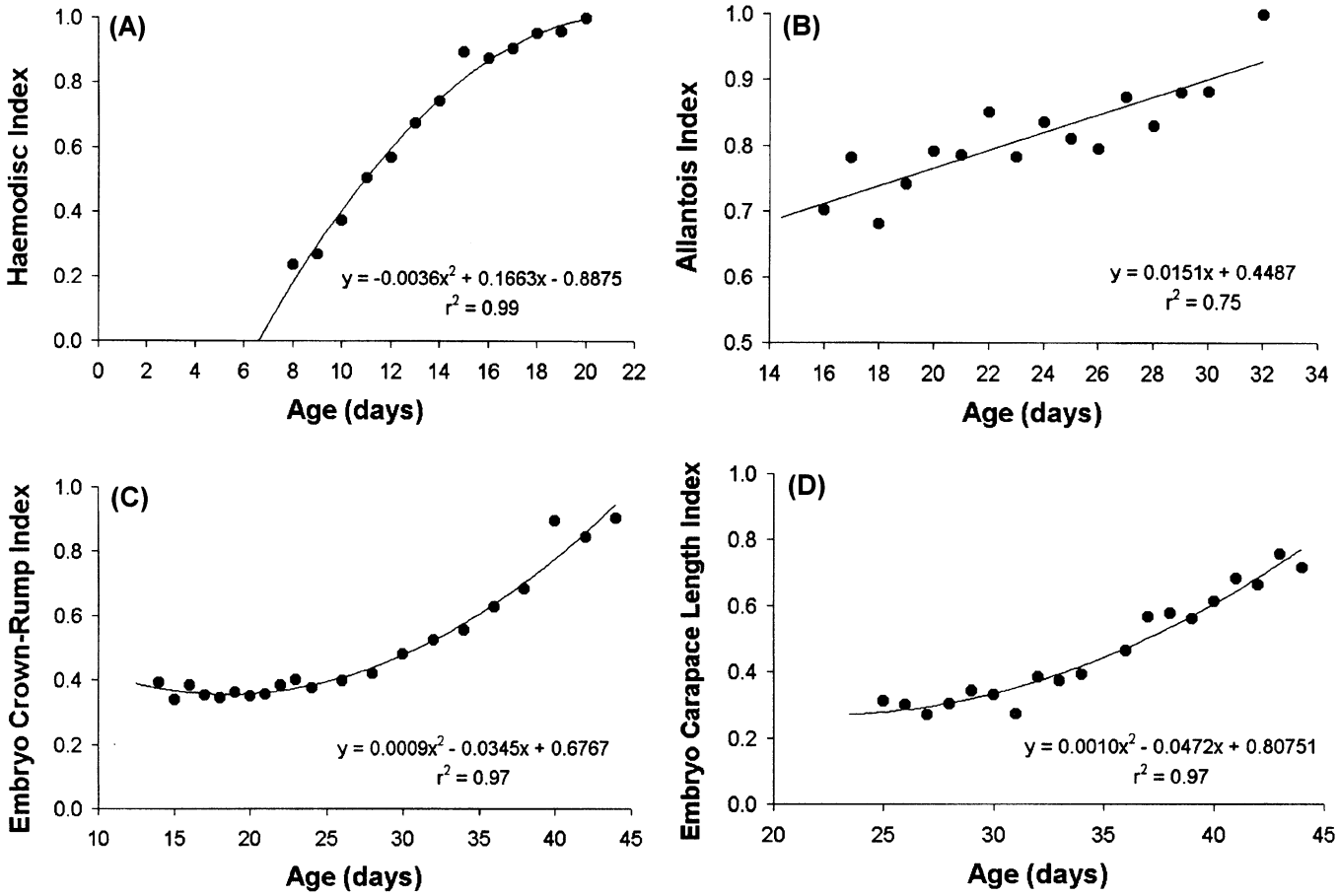


Fig. 11. Relationship between age (days) and haemodisc index (A), allantoic sac index (B), embryo crown–rump index (C), and embryo carapace-length index (D) for *C. insculpta* eggs incubated at 30°C. The regression equations and r^2 values are presented.



between stages 12 and 14, presumably because of an increase in embryo curvature rather than a reduction in length. It then remained constant until stage 16, after which it increased (Fig. 11C). Consequently, similar CRI values occurred at stages 12 and 17. Qualitative candling attributes readily distinguished between these stages, demonstrating the need for a combination of qualitative and quantitative data for effective staging from candling attributes. Using both, 18 stages could be distinguished using candling attributes (Tables 2 and 3).

Correction for incubation temperature

All experiments in this study (with the exception of 2 eggs incubated at 34°C in the patch experiment) were conducted at 30°C, which creates a problem in applying the results to ageing eggs incubated at other constant temperatures or in natural nests, where the mean temperature varies appreciably from 30°C. Webb et al. (1986) provided correction factors to allow conversion of results from one temperature to another. Using 30°C as a reference, Webb et al. (1986) reported that development was faster by a factor of 1.30 at 32°C and slower by a factor of 0.68 at 28°C. A. Georges (unpublished data cited in Beggs 1998)[2] has extended this work for *C. insculpta*, with additional observations shown in Table 4. These correction factors can be used to rescale the stage versus age observations, presented in this paper for 30°C, for application to other temperatures.

Discussion

Ewert (1985) first demonstrated the value of qualitative candling observations for ageing turtle embryos when, in a landmark study, he applied the technique to 37 species drawn from six families. However, in few studies have quantitative candling measurements been used for ageing embryos. In those where they were used, attention was restricted to measuring the spread of the opaque patch (Webb et al. 1983*a*, 1983*b*, 1986; Thompson 1985). The present study demonstrates the value of both qualitative and quantitative candling attributes for determining the age of eggs and embryos of *C. insculpta*. We were consistently able to distinguish 18 stages on the basis of candling attributes alone.

There were three main periods during development for which candling attributes were useful. In the period before an embryo or its membranes were evident from candling (0–2.5 d, before stage 5), ageing was possible using the extent of the opaque patch. From days 7 to 52 (stage 5–22), ageing could be done using qualitative embryo attributes evident

[2] K.E. Beggs. 1998. Embryonic development of the pig-nosed turtle, *Carettochelys insculpta*, under constant and fluctuating temperature regimes. B.Sc. (Hons.) thesis, University of Canberra, Canberra, Australia. p. 126.

Table 3. Quantitative measurements of *C. insculpta* embryos during normal development, based on candling eggs incubated at 30°C.

Stage	Age	Opaque-patch index (OPI)	Haemodisc index (HI)	Crown–rump index (CRI)	Carapace-length index (CLI)
	0–18 h	≤0.16	—	—	—
—	18–24 h	0.16–0.28	—	—	—
—	24–30 h	0.28–0.39	—	—	—
—	30–36 h	0.39–0.51	—	—	—
—	36–42 h	0.51–0.63	—	—	—
—	42–48 h	0.63–0.74	—	—	—
—	48–60 h	0.74–0.98	—	—	—
≤6*	2.5–8 d	≥0.98	—	—	—
7–8*	8–10 d	—	0.21–0.42	—	—
9–11*	10–14 d	—	0.42–0.74	—	—
12	14–15 d	—	0.74–0.80	0.37–0.36	—
13	15–18 d	—	0.80–0.94	0.36–0.35	—
14	18–20 d	—	0.94–1.00	0.35	—
15	20–22 d	—	≥1.00	0.35	—
16	22–25 d	—	—	0.35–0.38	—
17	25–29 d	—	—	0.38–0.43	0.25–0.28
18	29–32 d	—	—	0.43–0.49	0.28–0.32
19	32–36 d	—	—	0.49–0.60	0.32–0.40
20	36–41 d	—	—	0.60–0.78	0.40–0.55
21	41–46 d	—	—	0.78–0.99	0.55–0.75
22	46–52 d	—	—	≥0.99	≥0.75
23	52–59 d	—	—	—	—
24	59–66 d	—	—	—	—
25	66–77 d	—	—	—	—
26	≥77 d	—	—	—	—

Note: The range of each developmental index for each age-class was predicted using the regression equations shown in Fig. 11. Stage was verified by direct examination of embryos.

*No embryos were killed at these stages, therefore those shown are only estimates.

Table 4. Developmental rates and development-rate coefficients (DRCs) for *C. insculpta* embryos incubated at a range of temperatures.

Incubation temp. (°C)	Development rate (mm/d)	DRC
28	0.138	0.6864
30	0.201	1
32	0.2337	1.1628
34	0.4021	2.0004

Note: Development rate, measured as the rate of change of head width per day, was calculated from the predictive relationship derived by A. Georges (unpublished data). DRCs were calculated by scaling the development rate at each temperature by the rate at 30°C (after Webb et al. 1986).

when candling (Table 2) and candling measurements of the embryo (Table 3). It was primarily in the final stages of development (52–77 d, stages 23–26) that candling yielded little reliable information for ageing. Here one must rely on estimating the proportion of the egg occupied by the embryo and yolk, respectively, and on assessing whether or not the yolk has been internalised in preparation for hatching. A second window of obscurity lies between days 3 and 7, after the opaque patch loses its utility but before the blood islands appear. These two periods notwithstanding, the ability to accurately age embryos from qualitative candling attributes and measurements represents a significant advance not only for embryological studies on *C. insculpta*, but potentially for embryological or ageing studies of other oviparous reptiles.

Direct measurements of embryos also provided useful information for ageing. Strong predictive relationships between age/stage and HWR existed up to and including stage 24, after which HWR changed little. This confirms the observations of Webb et al. (1986) that in *C. insculpta*, embryo size reaches a maximum before qualitative changes are complete. During this late maturation phase, embryos underwent minor morphological changes involving the eyelids and pigmentation, and changes associated with yolk internalisation. Although growth in the index of embryo size, HWR, halted at stage 24, it is possible that other quantifiable attributes may have continued to change up to hatching (stage 26). For example, embryo length has been found to increase until development is complete in snapping turtles (Yntema 1968) and crocodilians (Deeming and Ferguson 1989). Similarly, embryo mass increases throughout development in turtles (Pieau and Dorizzi 1981; Ewert 1985) and crocodilians (Deeming and Ferguson 1989), although this is not always a reliable index of size, because mass can be confounded by embryonic water content and the amount of residual yolk (Piersma and Davidson 1991; Cagle et al. 1993). In light of these established trends, it seems likely that *C. insculpta*

embryos continue to grow between stages 24 and 26, but that this growth was not reflected in HWR measurements. Consequently, application of relationships among HWR, age, and stage for ageing was limited to the period of development up to stage 24. This limitation could be overcome in the relationship between stage and age, where the late developmental curvilinearity could be accommodated by a log-linear relationship and hence be made useful as a staging tool beyond stage 24. We support Miller's (1985*a*) call for further attention to be paid to embryo measurements in staging studies, as a complement to qualitative characters. Further work involving alternative growth indices is needed to identify the best measure of embryo size, preferably one that is linearly related to time, is not subject to great errors in its determination, and continues to increase throughout development.

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References

Billett, F.S., Collins, P., Goulding, D.A., and Sutherland, J. 1992. The development of *Caretta caretta*, at 25–34°C, in artificial nests. J. Morphol. **213**: 251–263.

Blake, D.K. 1974. The rearing of crocodiles for commercial and conservation purposes in Rhodesia. Rhod. Sci. News, **8**: 315–324.

Blanck, C.E., and Sawyer, R.H. 1981. Hatchery practices in relation to early embryology of the loggerhead sea turtle, *Caretta caretta* (Linné). J. Exp. Mar. Biol. Ecol. **49**: 163–177.

Bull, J.J., and Vogt, R.C. 1981. Temperature-sensitive periods of sex determination in emydid turtles. J. Exp. Zool. **218**: 435–440.

Cagle, K.D., Packard, G.C., Miller, K., and Packard, M.J. 1993. Effects of the microclimate in natural nests on development of embryonic painted turtles, *Chrysemys picta*. Funct. Ecol. **7**: 653–660.

Deeming, D.C., and Ferguson, M.W.J. 1989. Effects of incubation temperature on growth and development of embryos of *Alligator mississippiensis*. J. Comp. Physiol. B, **159**: 183–193.

Deeming, D.C., and Thompson, M.B. 1991. Gas exchange across reptilian eggshells. *In* Egg incubation: its effects on embryonic development in birds and reptiles. *Edited by* D.C. Deeming and M.W.J. Ferguson. Cambridge University Press, Cambridge. pp. 277–284.

Dufaure, J.P., and Hubert, J. 1961. Table de développement du lézard vivipare *Lacerta* (*Zootoca*) *vivipara* Jacquin. Arch. Anat. Microsc. Morphol. Exp. **50**: 309–328.

Ewert, M.A. 1985. Embryology of turtles. *In* Biology of the Reptilia. Vol. 14. *Edited by* C. Gans, F. Billett, and P.F.A. Maderson. John Wiley and Sons, New York. pp. 75–267.

Ewert, M.A., and Legler, J.M. 1978. Hormonal induction of oviposition in turtles. Herpetologica, **34**: 314–318.

Ferguson, M.W.J. 1985. The reproductive biology and embryology of crocodilians. *In* Biology of the Reptilia. *Edited by* C. Gans, F. Billett, and P.F.A. Madereson. John Wiley and Sons, New York. pp. 329–491.

Georges, A., and Kennett, R. 1989. Dry-season distribution and ecology of *Carettochelys insculpta* (Chelonia: Carettochelydidae) in Kakadu National Park, northern Australia. Aust. Wildl. Res. **16**: 323–335.

Georges, A., and Rose, M. 1993. Conservation biology of the pig-nosed turtle, *Carettochelys insculpta*. Chelonian Conserv. Biol. **1**: 3–12.

Guyot, G., Pieau, C., and Renous, S. 1994. Développement embryonnaire d'une tortue terrestre, la tortue d'Hermann, *Testudo hermanni* (Gmelin, 1789). Ann. Sci. Nat. Zool. Paris Ser. 13, **15**: 115–137.

Hubert, J. 1985. Embryology of the squamata. *In* Biology of the Reptilia. Vol. 15. *Edited by* C. Gans and F. Billett. John Wiley and Sons, New York. pp. 3–34.

Hubert, J., and Dufaure, J.P. 1968. Table de développement de la vipère aspic : *Vipera aspic*. Bull. Soc. Zool. Fr. **93**: 135–148.

Limpus, C.J., Baker, V., and Miller, J.D. 1979. Movement induced mortality of loggerhead eggs. Herpetologica, **35**: 335–338.

Magnusson, W.E., and Taylor, J.A. 1980. A description of developmental stages in *Crocodylus porosus*, for use in ageing eggs in the field. Aust. Wildl. Res. **7**: 479–485.

Mahmoud, I.Y., Hess, G.L., and Klicka, J. 1973. Normal embryonic stages of the western painted turtle, *Chysemys picta bellii*. J. Morphol. **130**: 479–489.

Miller, J.D. 1985*a*. Criteria for staging reptilian embryos. *In* Biology of Australasian frogs and reptiles. *Edited by* G. Grigg, R. Shine, and H.E. Ehmann. Australian Zoological Society of New South Wales, Sydney. pp. 305–310.

Miller, J.D. 1985*b*. Embryology of marine turtles. *In* Biology of the Reptilia. Vol. 14. *Edited by* C. Gans, F. Billett, and P.F.A. Maderson. John Wiley and Sons, New York. pp. 269–328.

Moffat, L.A. 1985. Embryonic development and aspects of reproductive biology in the tuatara, *Sphenodon puntatus*. *In* Biology of the Reptilia. Vol. 14. *Edited by* C. Gans, F. Billett, and P.F.A. Maderson. John Wiley and Sons, New York. pp. 493–522.

Parmenter, C.J. 1980. Incubation of the eggs of the green turtle, *Chelonia mydas*, in Torres Strait, Australia: the effect of movement on hatchability. Aust. Wildl. Res. **7**: 487–491.

Pieau, C., and Dorizzi, M. 1981. Determination of temperature-sensitive stages for sexual differentiation of the gonads in embryos of the turtle, *Emys orbicularis*. J. Morphol. **170**: 373–382.

Piersma, T., and Davidson, N.C. 1991. Confusions of mass and size. Auk, **108**: 441–444.

Raynaud, A. 1961. Quelques phases du développement des oeufs chez l'orvet (*Anguis fragilis* L.). Bull. Biol. Fr. Belg. **65**: 365–382.

Renous, S., Rimblot-Baly, F., Fretey, J., and Pieau, C. 1989. Caractéristiques du développement embryonnaire de la Torte luth, *Dermochelys coriacea* (Vandelli, 1761). Ann. Sci. Nat. Zool. Paris Ser. 13, **10**: 197–229.

Thompson, M.B. 1985. Functional significance of the opaque white patch in eggs of *Emydura macquarii*. *In* Biology of Australasian frogs and reptiles. *Edited by* G. Grigg, R. Shine, and H.E. Ehmann. Australian Zoological Society of New South Wales, Sydney. pp. 387–395.

Webb, G.J.W., Buckworth, R., and Manolis, S.C. 1983*a*. *Crocodylus johnstoni* in the McKinlay River, N.T. VI. Nesting biology. Aust. Wildl. Res. **10**: 607–637.

Webb, G.J.W., Buckworth, R., Sack, G.C., and Manolis, S.C. 1983*b*. An interim method for estimating the age of *Crocodylus porosus* embryos. Aust. Wildl. Res. **10**: 563–570.

Webb, G.J.W., Choquenot, D., and Whitehead, P. 1986. Nests, eggs and embryonic development of *Carettochelys insculpta* (Chelonia: Carettochelidae) from northern Australia. J. Zool. Ser. B, **1**: 512–550.

Webb, G.J.W., Beal, A.N., Manolis, S.C., and Dempsey, K.E. 1987. The effects of incubation temperature on sex determination and embryonic development rate in *Crocodylus johnstoni* and *C. porosus*. *In* Wildlife management: crocodiles and alligators. *Edited by* G.J.W. Webb, S.C. Manolis, and P.J. Whitehead. Surrey Beatty and Sons, Chipping North, New South Wales. pp. 507–534.

Yntema, C.L. 1968. A series of stages in the embryonic development of *Chelydra serpentina*. J. Morphol. **125**: 219–252.

Yntema, C.L. 1979. Temperature levels and periods of sex determination during incubation of eggs of *Chelydra serpentina*. J. Morphol. **159**: 17–27.

Zehr, D.R. 1962. Stages in the normal development of the common garter snake, *Thamnophis sirtalis sirtalis*. Copeia, 1962: 322–329.