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Staging Criteria for Embryos of the Spiny Softshell Turtle, *Apalone spinifera* (Testudines: Trionychidae)

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ABSTRACT Previous work describing the embryonic stages of turtle development has not included members of the highly derived trionychid turtles. Staging criteria are described for the spiny softshell turtle (*Apalone spinifera*) to facilitate comparisons between phylogenetically distant taxa of turtles. Embryonic development in *A. spinifera* is placed in the context of the widely used sequence of Yntema stages. Novel features are included in the descriptions of staging criteria for Stages 13–26. Comparisons of the development of specific features are made between *A. spinifera* and other taxa of turtles. Data on the duration of developmental stages at different temperatures and embryo dimensions support the conclusion that morphology-based staging criteria are superior to developmental rate temperature coefficients. J. Morphol. 254:272–291, 2002.

KEY WORDS: Testudines; Trionychidae; *Apalone spinifera;* embryology

Prior to formal description and standardization of the embryonic stages of development in turtles, researchers used measurement ranges to describe approximate ages of embryos (e.g., Risley, 1933). Yntema (1968) introduced the use of a normative series of developmental stages for turtle embryos with his description of 27 stages (0–26) in the development of *Chelydra serpentina*. His stages were based on timed periods of development at a given constant temperature. For each stage he provided a detailed description of the external morphology and suggested that some of the characteristics would serve as differential criteria for stage recognition. Since the publication of Yntema's (1968) staging criteria, several other normal series have been described for various turtles; however, this proliferation has tended to complicate rather than facilitate making taxonomic comparisons. For example, in their study of the emydid turtle *Chrysemys picta,* Mahmoud et al. (1973) described 23, rather than 27, developmental stages. Crastz (1982) described 31 developmental stages in the cheloniid *Lepidochelys olivacea* based on morphology and measurements of embryos. Miller (1985) departed from prior authors by including preovipositional stages among his 31 stages of development in several taxa of embryonic sea turtles (*Chelonia mydas, Natator depressa, Caretta caretta, Eretmochelys imbricata, Lepidochelys olivacea*, and *Dermochelys coriacea*), a scheme subsequently followed by Renous et al. (1989) for *D. coriacea* and Billett et al. (1992) for *C. caretta*.

The seminal work of Ewert (1985) on turtle embryology employed the stages of Yntema (1968) for descriptive and comparative purposes, including considerable effort to interpret the older, classic studies of descriptive embryology in terms of Yntema's stages. Ewert (1985) extended the use of the Yntema criteria to include particular stages with highly visible characteristics that could be discerned by candling of eggs, thus allowing approximate aging of embryos without their sacrifice. This approach was elaborately developed for *Carettochelys insculpta* in concert with a summary normal series in terms of Yntema stages (Beggs et al., 2000). Many studies have routinely aged turtle embryos using Yntema stages, such as various reproductive studies (e.g., Pieau and Dorizzi, 1981; Raynaud and Pieau, 1985; Wibbels et al., 1991). In addition, Guyot et al. (1994) described a normal series using Yntema-equivalent stages for the tortoise *Testudo hermanni*.

In order to facilitate comparison of structures or events in the development of any related group of organisms, a standard set of developmental stages is essential. Although Yntema's (1968) staging series is widely used for turtles, trionychids lack some key morphological features owing to their highly divergent morphology. For example, carapacial scutes are absent and there is a general reduction of keratinized integumentary structures, including the presence of only three digits with claws per limb. Some prominent attributes of trionychids have never been used in an embryonic staging series (e.g., lips along the upper and

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lower jaws), but there are few published studies involving embryonic trionychids. Ling et al. (1985) did not stage embryos of the Asian trionychid *Pelodiscus sinensis* in their study of epiplexus cells; embryonic age (in days) was used in place of a staging scheme. Cherepanov (1995) referred to ranges of Yntema stages (e.g., 18–21) in a study of shell development in the same species. Webb et al. (1986) attempted to stage embryos of *Carettochelys insculpta,* the sister group of the trionychids (Shaffer et al., 1997), with metric data (e.g., head measurements) that are only vaguely comparable to Yntema stages. Beggs et al. (2000) devised a *Carettochelys* staging series meant to coincide with that of Yntema (1968) and they described characters taking into account its unique morphology, some of which are shared with trionychids, e.g., the lack of carapacial scutes.

Herein, we describe an objective scheme of morphological stages for the trionychid *Apalone spinifera* meant to correspond with the Yntema series. We assume that the earliest developmental stages are common to all turtles (based on fundamental features found in all turtle taxa); therefore, we emphasize the second half of embryonic development, during which time the distinctive trionychid features are formed. Another objective is to compare the several published normal series for turtles and development of selected features across taxa.

MATERIALS AND METHODS

A total of 306 eggs of *Apalone spinifera* was collected for the purpose of this study. Forty-four eggs (collected from Big Bayou Sara and Little Bayou Sara, West Feliciana Parish, Louisiana, in the second half of May 1996, hereafter denoted 96A) and 46 eggs (collected from nests or gravid females from Ouachita Parish, hereafter denoted 96B) were maintained at a steady temperature of 24.5°C until 10 June 1996, when all eggs were moved to an incubator. Thirty-six eggs were obtained from nests or gravid females from Black Bayou Lake in Ouachita Parish, Louisiana, in May and June 1997 (hereafter denoted 97A) and an additional 180 eggs were obtained on 4 July 1997 from Concordia Turtle Farm in Wildsville, Louisiana (hereafter denoted 97B). All eggs were individually numbered and placed in plastic boxes (4.5 × 15 × 23 cm) containing a 1:1 mixture of granular vermiculite to distilled water by weight.

The clutches from 1996 (96A, B) and the wild-caught clutches from 1997 (97A) were incubated at 31°C. The farm-collected clutches from 1997 (97B) were divided randomly and placed into incubators on 9 July 1997 at 31°C or 10 July 1997 at 26°C (following the methods of Bull et al., 1988). Eggs 96A and B were incubated in a standard cabinet incubator (Precision Scientific Lab Incubator). The 1997 eggs were placed in incubators constructed from insulation board (60 × 70 × 74 cm) glued together with silicone (Lang et al., 1989). Plastic boxes containing the eggs were placed on a metal shelf over a temperature-regulated container of water inside the incubator. Eggs were checked for chalking, vitelline circulation (Ewert, 1985; Beggs et al., 2000), and other signs of development at least weekly, creating some exchange of air in the incubator. A digital thermostat (Helix Controls, San Diego, CA) maintained the water temperature, and thereby the internal incubator temperature, to within 0.1°C. The temperatures of the incubators fluctuated no more than 0.6°C from the desired setpoints of 26°C and 31°C, respectively.

Webb (1962) referred to all populations of *Apalone spinifera* from the lower Mississippi River Valley as intergrades of several subspecies, the ranges of which meet in the area. The specimens from West Feliciana Parish are consistent with populations that Webb considered intermediate in characterization between *A. s. spinifera* and *A. s. hartwegi*, with some possible influence of *A. s. aspera*. Ouachita Parish specimens apparently are intergrades between *A. s. spinifera* and *A. s. hartwegi* and those from Concordia Turtle Farm exhibited somewhat more *A. s. spinifera* influence. We expect that the morphological characteristics mentioned in this work are valid for all subspecies of *A. spinifera*, with the possible exception of carapace pigmentation in *A. s. pallida, A. s. guadalupensis*, and *A. s. emoryi*.

Embryos incubated in 1996 (96A, B) were sacrificed starting on 26 June 1996 by severing the spinal cord. Embryos from 1997 (97A, B) were sacrificed by immersion in a 0.2% solution of MS-222 if they were less than 1 cm in total length, or by intracardiac injection of sodium pentobarbital if they had a total length exceeding 1 cm (AVMA Panel on Euthanasia, 1993). Embryos from 1996 (96A, B) were preserved in 10% formalin for 12 months before transfer to Bouin's solution for at least 2 weeks, then stored in 50% isopropyl alcohol (Presnell and Schreibman, 1997). Embryos from 1997 (97A, B) were preserved in Bouin's solution for at least 2 weeks before transfer to 50% isopropyl alcohol for long-term storage. A total of 32 embryos was harvested from the 1996 clutches and 101 embryos were collected from the 1997 (97A, B) eggs. Several turtles were allowed to hatch from each clutch of eggs (to be used in later studies). The remaining eggs were either infertile or the embryos died in situ due to unknown causes.

Embryos from Stages 11–15 were definitively staged according to Yntema's (1968) criteria. Embryos beyond these stages were classified with morphological characters including backlit claw structure, nasal structures, urogenital papilla development, labial development, and nictitating membrane appearance. Claw structure is described from the second digit in dorsal view, as seen by shining a bright light through the translucent digit. The duration of stage for each temperature was calculated by plotting each staged embryo on a timeline (in days) for each clutch of eggs separately for each temperature. We divided the elapsed time equally among the interim stages and assigned that value to each embryo for those stages involved. The average duration of each stage was calculated by obtaining the mean of all embryo-specific stage durations. Following stage classification, embryos were photographed and placed in 50% isopropyl alcohol. The kidney/gonad complex was extracted for use in a companion study of gonadogenesis (Greenbaum and Carr, 2001). Head and carapace lengths of the embryos were measured with dial calipers to the nearest 0.05 mm. Carapace length measurements before Stage 16 were not taken because the carapace is not fully formed until this stage.

RESULTS

The following results are based on examination of 112 embryos of *Apalone spinifera*; 47 were incubated at 26°C and 65 at 31°C. At 26°C, embryos ranged from Stages 11–26, whereas at 31°C embryos examined ranged from Stages 12 and 14–26.

Description of Stages

Through Stage 23, we present the staging criteria used by Yntema that are pertinent to *Apalone spinifera* first, followed by a description of novel features (unique to *A. spinifera*) we found useful for staging. The latter are comparable to those used by Yntema (1968) to assign stages to *Chelydra serpentina*. We follow Yntema's terminology in describing the frontal, mandibular, maxillary, and nasal processes of younger embryos. The "processes" refer to the most anterior points of the developing frontal bone, lower jaw, upper jaw, and proboscis of the embryo, respec-

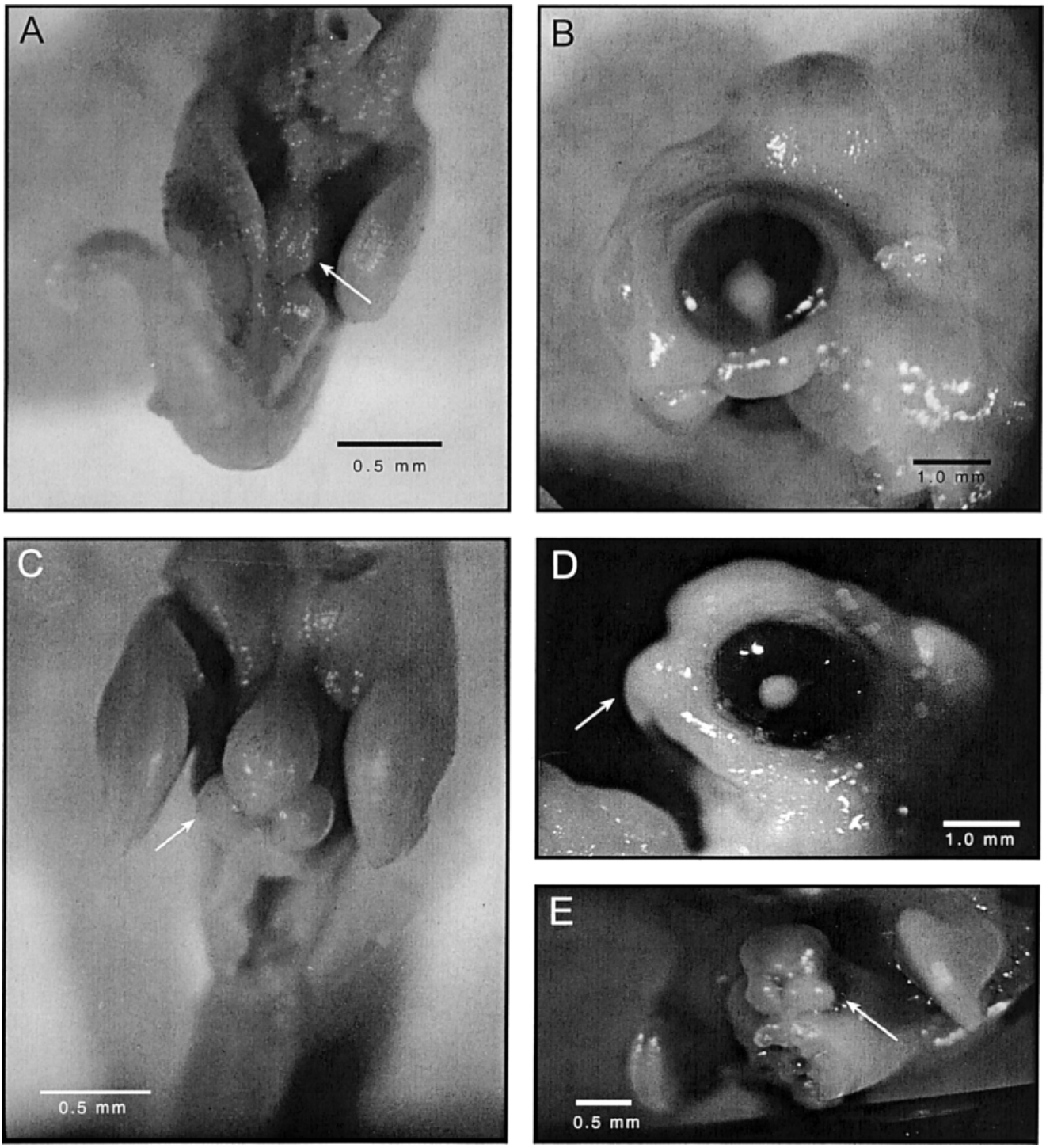

Fig. 1. Photomicrographs of embryonic structures in *Apalone spinifera.* **A:** Ventral view of an incipient urogenital papilla, Stage 13 embryo. **B:** Lateral view of the head of a Stage 14 embryo; note that the nasal process is just slightly anterior to the frontal process. **C:** Ventral view of an incipient urogenital papilla of a Stage 14 embryo. **D:** Lateral view of the head of a Stage 15 embryo; the nasal process (arrow) is clearly more anterior than the frontal process and the anterior margin of the mandibular process extends to the anterior edge of the pupil. **E:** Ventral view of the urogenital papilla of a Stage 15 embryo; note the crease of the forming vent around the periphery of the structure.

tively. Emphasis is on external characteristics in staging whole embryos, as illustrated in Figures 1–13.

Stage 11. The first pharyngeal slit is open dorsally and the second slit is covered by the hyoid arch. The fifth pharyngeal arch is conspicuous posteriorly

and the maxillary process extends toward the eye. The eye lacks retinal pigmentation (Yntema, 1968).

Stage 12. The pharyngeal slits are not visible. The maxillary process extends as far ventrally as the mandibular process. The hyoid arch is visible. The retina is black. The forelimb bud is slightly shorter than wide. The axis of the limb is oriented posteroventrally and has an inchoate apical ridge (Yntema, 1968).

Stage 13 (Fig. 12A). The maxillary process extends beyond the mandibular process and posteriorly delimits a nasolacrimal groove that can be traced to the olfactory pit. The forelimb bud is slightly longer than wide, with its axis more caudal than ventral. The apical ridge is distinct (Yntema, 1968). A small protuberance (the primordium of the urogenital papilla) is present on the ventral surface of the tail between the hindlimbs (Fig. 1A).

Stage 14 (Fig. 12B). The maxillary process and lateral part of the nasal process are connected. The mandibular process is less distinct than in the previous stage (Fig. 1B). The forelimb axis is oriented caudally and bears indications of a digital plate. A slight dorsolateral groove marks the incipient lateral border of the carapace (Yntema, 1968). The protuberance forming the primordium of the cloaca and urogenital papilla is larger than in the previous stage and composed of three distinct lobes (Fig. 1C).

Stage 15 (Fig. 12C). The digital plate of the forelimb bud is conspicuous, but lacks digital grooves. The lateral part of the carapace is clearly delimited. A loop of the gut is herniated through the incipient plastron (Yntema, 1968). The anterior edge of the lower jaw is located at the level of the anterior edge of the pupil. The nasal area protrudes slightly anterior to the frontal process border (Fig. 1D). An incipient vent surrounds the large, rounded eminence at the base of the tail; at this stage, the eminence can be identified as the urogenital papilla (Fig. 1E).

Stage 16 (Fig. 12D). Scleral papillae are evident in some specimens. The digital plate of the forelimb is larger, smooth around the periphery, and bears slight indications of digital ridges. The anterior margin of the carapace is evident (Yntema, 1968). Maxillary and mandibular labia are present along the upper and lower jaws (Fig. 2A,C). The anterior margin of the lower jaw is located at the level of the anterior margin of the lens (Fig. 2A). In slightly older individuals of this stage (denoted as 16+), the occipital lobe is clearly bifurcated along the midline of the embryo's body; this bifurcation varies from a slight indentation to a conspicuous dark line (Fig 2B). The urogenital papilla projects from the vent (Fig. 2D).

Stage 17 (Fig. 12E). The periphery of the digital plate of the forelimb is slightly serrated and the incipient digits are marked by a series of four furrows that separate the five digital ridges (Yntema, 1968). The occipital protuberance and frontal process are subequal in height (Fig. 3A). As seen in ventral view, a slight horseshoe-shaped gap separates the upper and lower jaws (Fig. 3B). The carapace is pigmented with small black spots in some individuals.

Stage 18 (Fig. 12F). The lower eyelid is clearly evident (Fig. 4A). The digital plate bears distinct digits that protrude beyond the margin and form deep serrations in the periphery of the plate (Yntema, 1968). The frontal process now slightly exceeds the occipital protuberance in height (Fig. 4A). As seen in ventral view in most individuals, the upper and lower jaw closure is complete (Fig. 4B). A slight protuberance (genesis of the caruncle) is present on the ventral surface of the upper jaw (Fig. 4C). Under high magnification, inchoate spines can be seen on the carapace of some individuals.

Stage 19 (Fig. 12G). The lower eyelid nearly reaches the level of the scleral papillae and the second digit of the forelimb bud projects beyond the webbing a distance slightly greater than its thickness at the level of the web (Yntema, 1968; Fig. 5A,D). The caruncle and the spines on the carapace are more prominent than in the previous stage (Fig. 5B,C). There is little differentiation between the digits and the webbing (Fig. 5D). Incipient folds on the preaxial–dorsal area of the forelimb are present.

Stage 20 (Fig. 12H). The lower eyelid reaches the level of the lens (Fig. 6A) and the claw of the central digit of the forelimb bud projects beyond the web a distance about twice as great as its width at the web (Yntema, 1968). The longitudinal maxillary crease of the maxillary labium is absent or incipient; however, if present, the maxillary crease does not extend anteriorly beyond the anterior margin of the eye (Fig. 6A). The anterior margin of the frontal process recedes posteriorly (Fig. 6A). The occipital protuberance decreases in size to the point of no longer being distinct (Fig. 6A). A prominent groove between the base of each claw and the webbing is present on the forelimb bud (Fig. 6B) and the claws are opaque. In dorsal view, the webbing of Digits IV and V of the forelimbs is extensive and conceals the digits almost entirely (Fig. 6B). The preaxial–dorsal folds of the forelimbs are conspicuous (Fig. 6B). The urogenital papilla protrudes from the vent in all individuals (Fig 6C). In slightly older individuals (Stage 20+), the nictitating membrane is evident at the anterior corner of the eye (Fig. 6D).

Stage 21 (Fig. 13A). The lower eyelid overlaps the lower margin of the lens (Yntema, 1968; Fig. 7A). Scleral papillae are absent (Fig. 7A). The longitudinal maxillary crease varies from incipient to extending anteriorly to the most ventral point of the proboscis curvature. The forelimb claws are virtually opaque; however, a slight outline of the ungual phalanx is visible in some individuals (Fig. 7B). The urogenital papilla either projects from the vent or is completely withdrawn into the cloaca (Fig. 7C).

Stage 22 (Fig. 13B). The lower eyelid covers most of the pupil (Yntema, 1968; Fig. 7D). The longitudinal maxillary crease extends to the most ventral point of the proboscis curvature in all individuals.

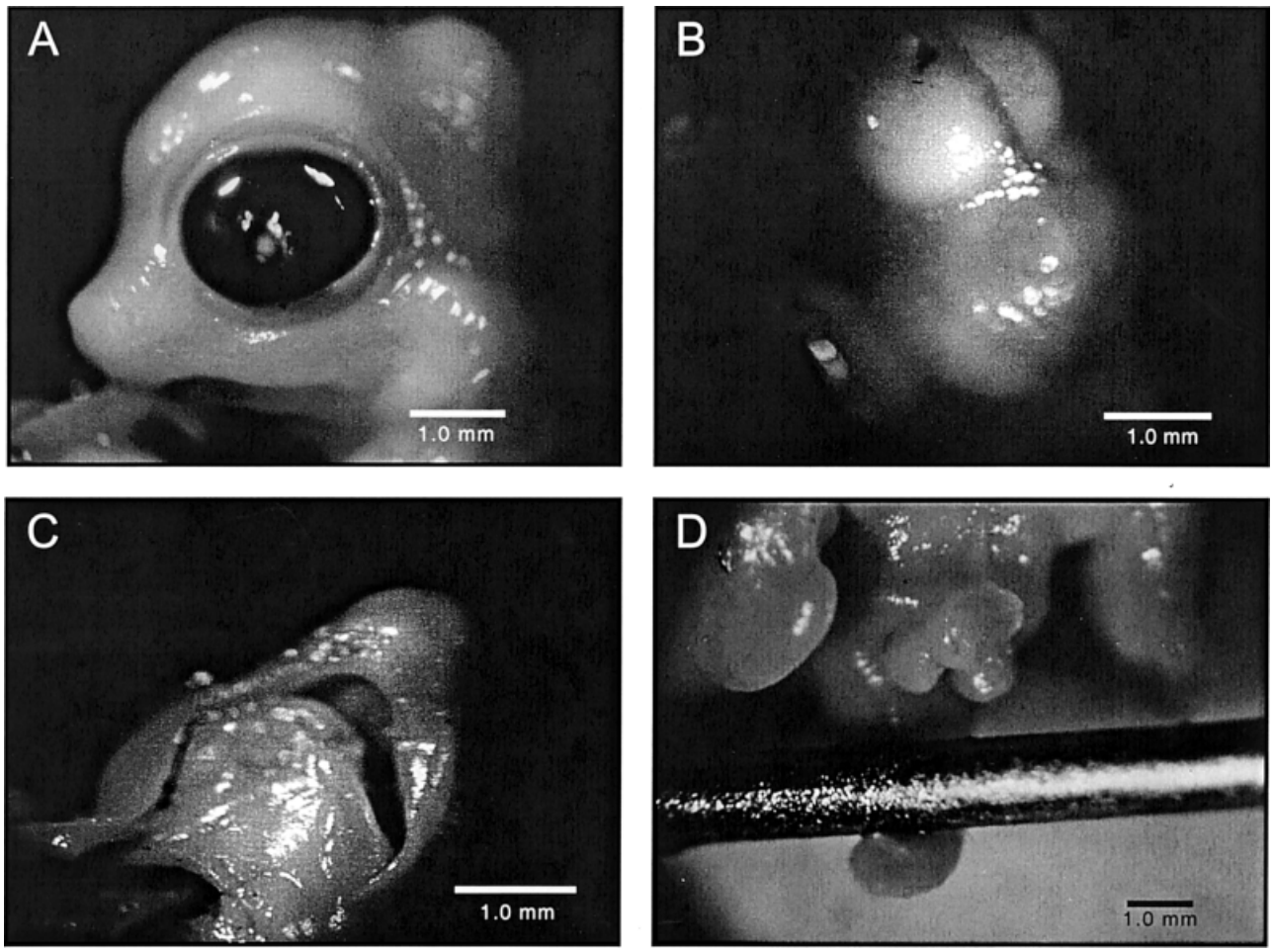

Fig. 2. Photomicrographs of embryonic structures in Stage 16 *Apalone spinifera.* **A:** Lateral view of the head. Notice that the lower jaw extends to the anterior margin of the lens; slight indications of lips are present and the proboscis is becoming increasingly pointed. **B:** Dorsal aspect of the occipital protuberance of a Stage 16+ embryo; a conspicuous bifurcation is present down the midline. **C:** Underside of the mouth; although well developed, the lower jaw has not yet extended the entire length of the upper jaw and lips can be seen on the lateral aspects of the upper and lower jaws. **D:** Ventral view of the urogenital papilla; note the three-lobed appearance of the structure.

There is a faint outline of the blunt ungual phalanx inside the claw of the forelimb; the apex of the bone lies proximal to the apex of the claw (Fig. 7E). The urogenital papilla is either visible inside the cloaca or completely withdrawn into the sealed vent (Fig. 7F).

Stage 23 (Fig. 13C). The upper and lower eyelids are separated by a narrow slit (Fig. 8A) and the loop of the gut that has been herniated is now retracted (Yntema, 1968). The external nares are sealed shut, but there is a slight demarcation of a ventral crease in the same plane as the internarial septum (Fig. 8C). The structure of the ungual phalanx is easier to see through a more translucent claw and may be more tapered toward the apex than at Stage 22 (Fig. 8B). Individuals with a blunt bone inside the claw and a narrow eyelid slit are designated Stage 23^{-}. The cloacal orifice (vent) is sealed in all individuals and the urogenital papilla is not visible. Deeply invaginated tissue surrounds the vent (Fig. 8D).

Stage 24 (Fig. 13D). The external nares are open at the tip of the proboscis, revealing long tubercles that protrude laterally from the internarial septum. The tubercles are oval when viewed through the narial openings, blunt apically, and extend beyond the radius of the circular narial cavity (Fig. 9B). The ungual phalanx has a tapered apex; no specimens have a blunt ungual phalanx at this stage.

Stage 25 (Fig. 13E). The narial tubercles flare anterodorsally and taper distally and each is shorter than the radius of the circular narial opening (Fig. 10B). The ungual phalanx tapers at a point more proximal to the webbing than at Stage 24. The claw is longer; therefore, the distance between the apex of the claw and the tip of the bone is slightly greater (Fig. 10C).

Stage 26 (Fig. 13F). In dorsal aspect, the distance from the apex of the ungual phalanx to the apex of the second claw is greater than, or equal to, the width of the claw at the apex of the bone. Each claw on the

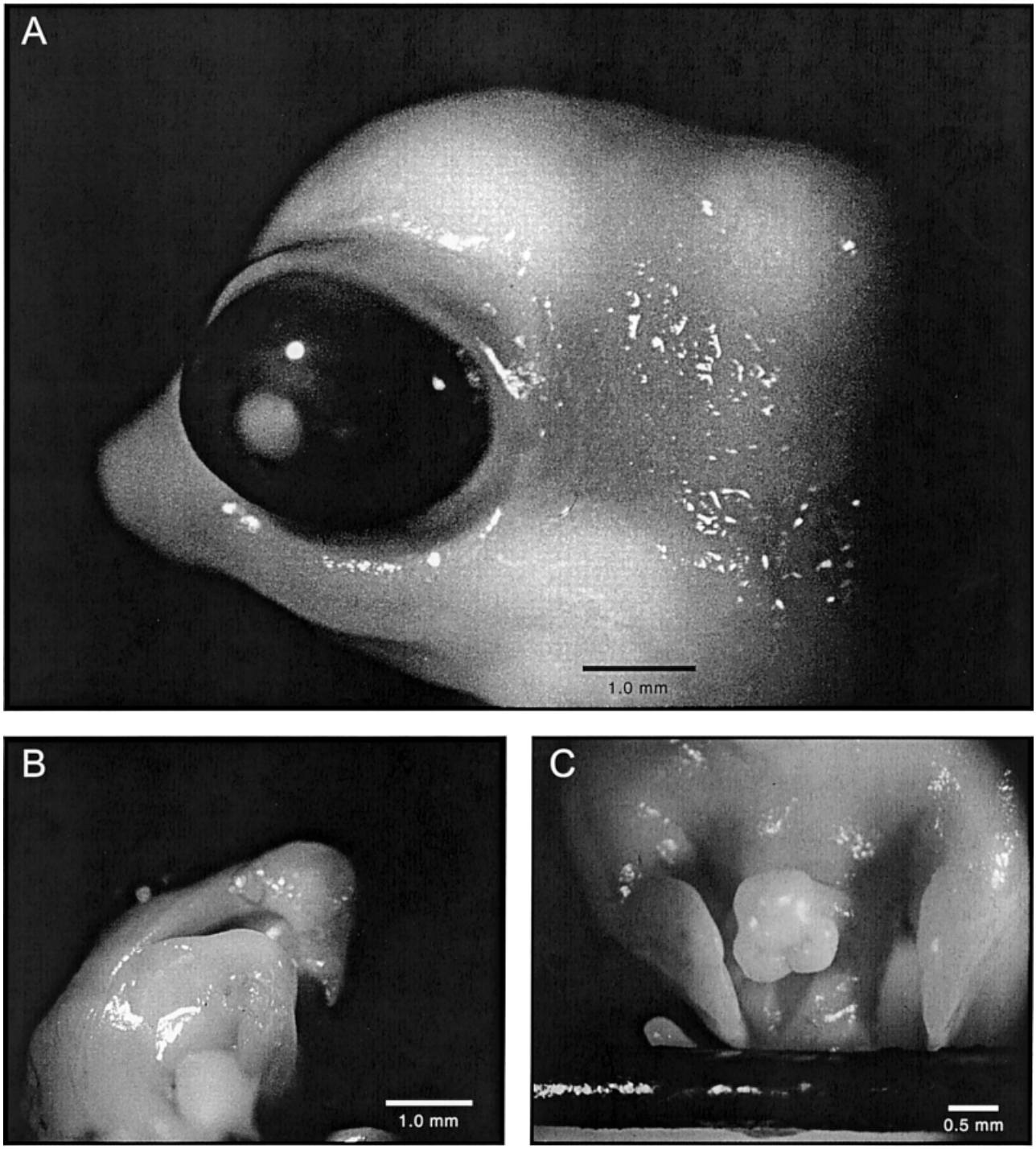

Fig. 3. Photomicrographs of embryonic structures in Stage 17 *Apalone spinifera.* **A:** Lateral view of the head; note that the occipital protuberance and frontal process are about equal in height. **B:** Underside of the mouth; closure with the upper jaw incomplete. **C:** Ventral view of the urogenital papilla.

forelimb is translucent at its apex and periphery (Fig. 11B). The claw becomes more translucent at its periphery and becomes increasingly flattened dorsoventrally as the embryo approaches hatching (Fig. 11B). Pigment on the base of the claw is extensive. A small umbilical hernia is present. Just prior to hatching (Stage 26+), the umbilical hernia is absent and a soft spot in the plastron is present in its place (Fig. 11C).

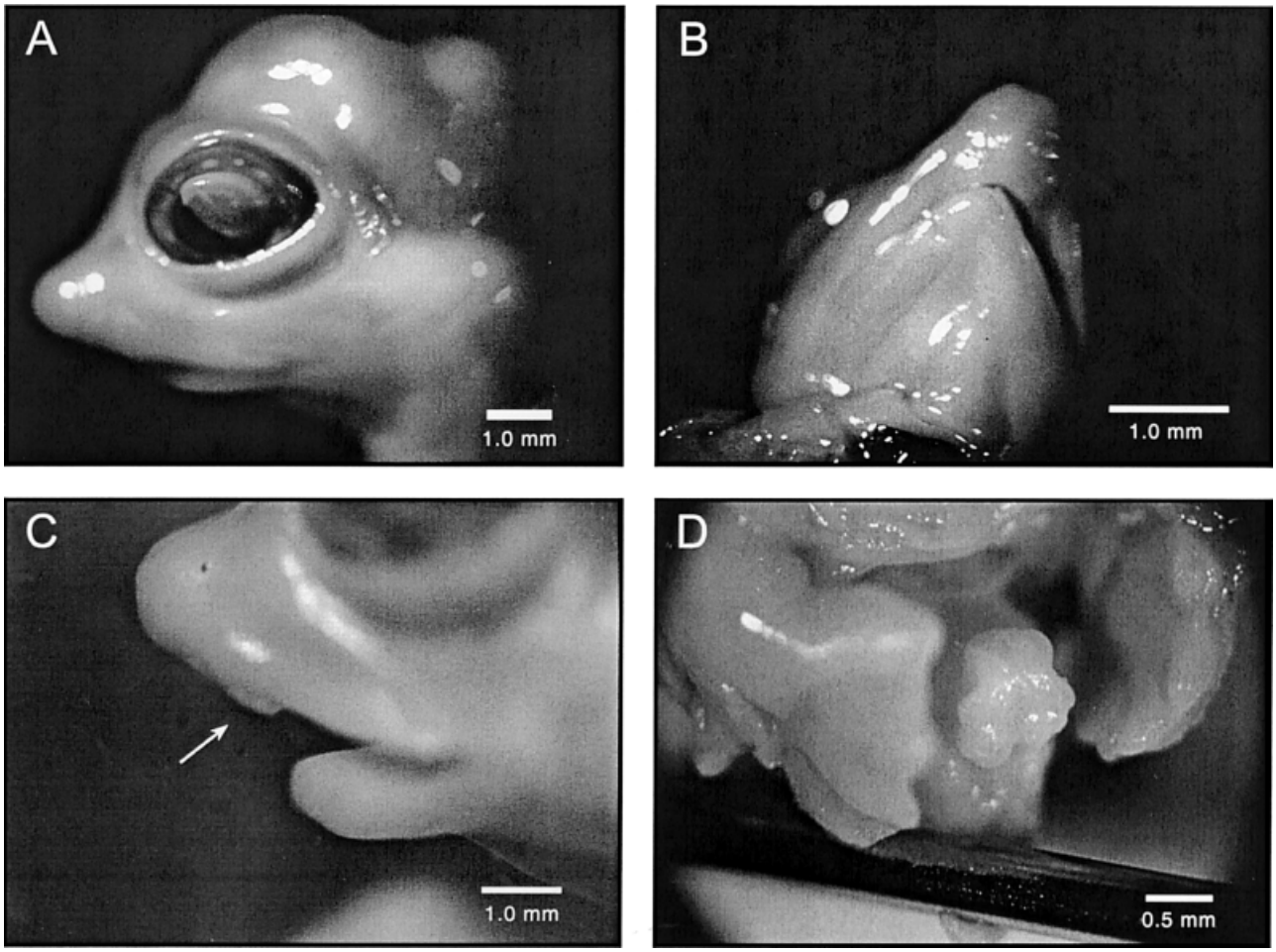

Fig. 4. Photomicrographs of embryonic structures in Stage 18 *Apalone spinifera.* **A:** Lateral view of the head; note that the occipital protuberance is shorter than the frontal process. Although they first appear in Stage 16 embryos, this specimen clearly shows scleral papillae. Note the indication of a lower eyelid. **B:** Underside of the mouth; closure of the lower jaw with the upper jaw is complete. **C:** Caruncle; the structure is incipient at this stage. **D:** Ventral aspect of the urogenital papilla.

Duration of Stages

The duration of each stage for 26°C and 31°C is shown in Table 1. At 26°C, the duration of an Yntema stage varied from 4–7 days. The corresponding range at 31°C was 2.3–6.3 days. For all stages (except Stage 22), development from one stage to the next took longer at 26°C than at 31°C. At 26°C, there is a general trend toward an increase in duration of each stage through Stage 21, after which there is a slight decline in duration. This is followed by a plateau from Stages 23–25 and then a marked increase in duration for Stage 26; however, our sample size for each stage is small. At 31°C, the trend is toward an increase of duration in each stage through Stage 22, at which time there is a decline in duration for Stage 23, followed by a plateau for the remaining stages.

Embryo Dimensions

Measurements of head width and carapace length of all embryos are shown in Table 2. Carapace lengths prior to Stage 16 were not measured because the periphery is not yet demarcated (Yntema, 1968). As a combined dataset, head width and carapace length generally increase as stages advance through Stage 25. An artifact of small sample size probably produced the slight decrease at Stage 26. At 26°C, head width increased until Stage 17, at which time there was an overall decrease (possibly an artifact of small sample size). No change in head width occurred between Stages 18 and 19; there was a decrease between Stages 22 and 24 (possibly an artifact of small sample size) and no change occurred between Stages 25 and 26. The carapace length at this temperature decreased from Stages 16–17, 22–24, and 25–26; again, these decreases may reflect small sample sizes. At 31°C, head width increased by stage until Stage 18. Small samples may have produced the decrease in head width from Stages 20–21 and 25–26. The carapace length at this temperature increased steadily through Stage 25, followed by a slight decrease at Stage 26. Overall size

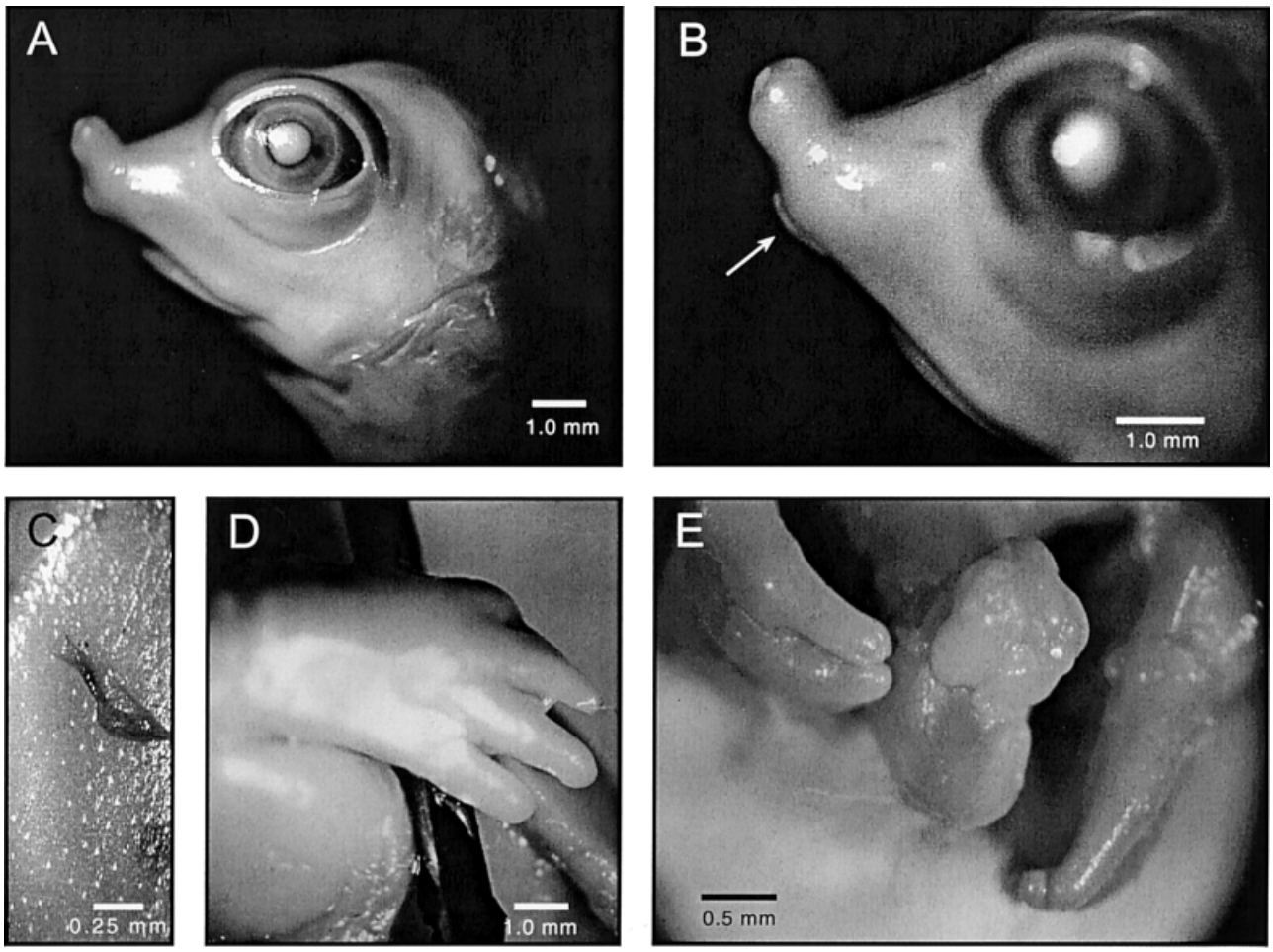

Fig. 5. Photomicrographs of embryonic structures in Stage 19 *Apalone spinifera.* **A:** Lateral view of the head; note that the lower eyelid has reached the level of the lens. **B:** Caruncle; the structure is well developed just under the proboscis of this individual. **C:** Carapace; spines are visible at high magnification on all individuals at this stage. **D:** Claw; the delineation of borders between claws and webbing is indistinct or absent. **E:** Ventral aspect of the urogenital papilla.

as measured by carapace length indicates that by the time of hatching, the warmer temperature produces larger hatchlings.

DISCUSSION

The staging scheme constructed for *Apalone spinifera* was designed to be as congruent as possible with Yntema's (1968) criteria for *Chelydra serpentina*; these have become nearly standard for comparative studies of turtle embryos that require a baseline set of morphologically determined chronological stages. As evidenced by the observations at Stages 11 and 12, the embryos of *A. spinifera* are similar to those of other species at early stages of development.

Comparison of Staging Schemes

In order to facilitate comparisons of 27-stage schemes (this study; Yntema, 1968; Raynaud and Pieau, 1985; Guyot et al., 1994; Beggs et al., 2000) with the 23-stage scheme of *Chrysemys picta* (Mahmoud et al., 1973) and 31-stage schemes of *Lepidochelys olivacea* and other sea turtles (Crastz, 1982; Miller, 1985; Renous et al., 1989), we applied the Yntema (1968) morphological criteria to these descriptions. Comparisons of staging schemes across taxa are obfuscated by disparate morphologies, heterochrony, and possible homoplasy. To minimize the subjectivity of our comparisons, we stressed morphological features that were easily comparable across taxa. These features, in decreasing order of importance, were forelimb morphology, eye morphology, shell morphology, and pigmentation. In the absence of a staging scheme that can be used for all turtle taxa, our comparisons must be considered tentative; perceived differences may be a result of character biases in staging schemes and not heterochrony. The resulting reference system allows comparison of any turtle stage from the above studies with its equivalent Yntema (1968) stage (Table 3). Renous et al. (1989) attempted a similar table of

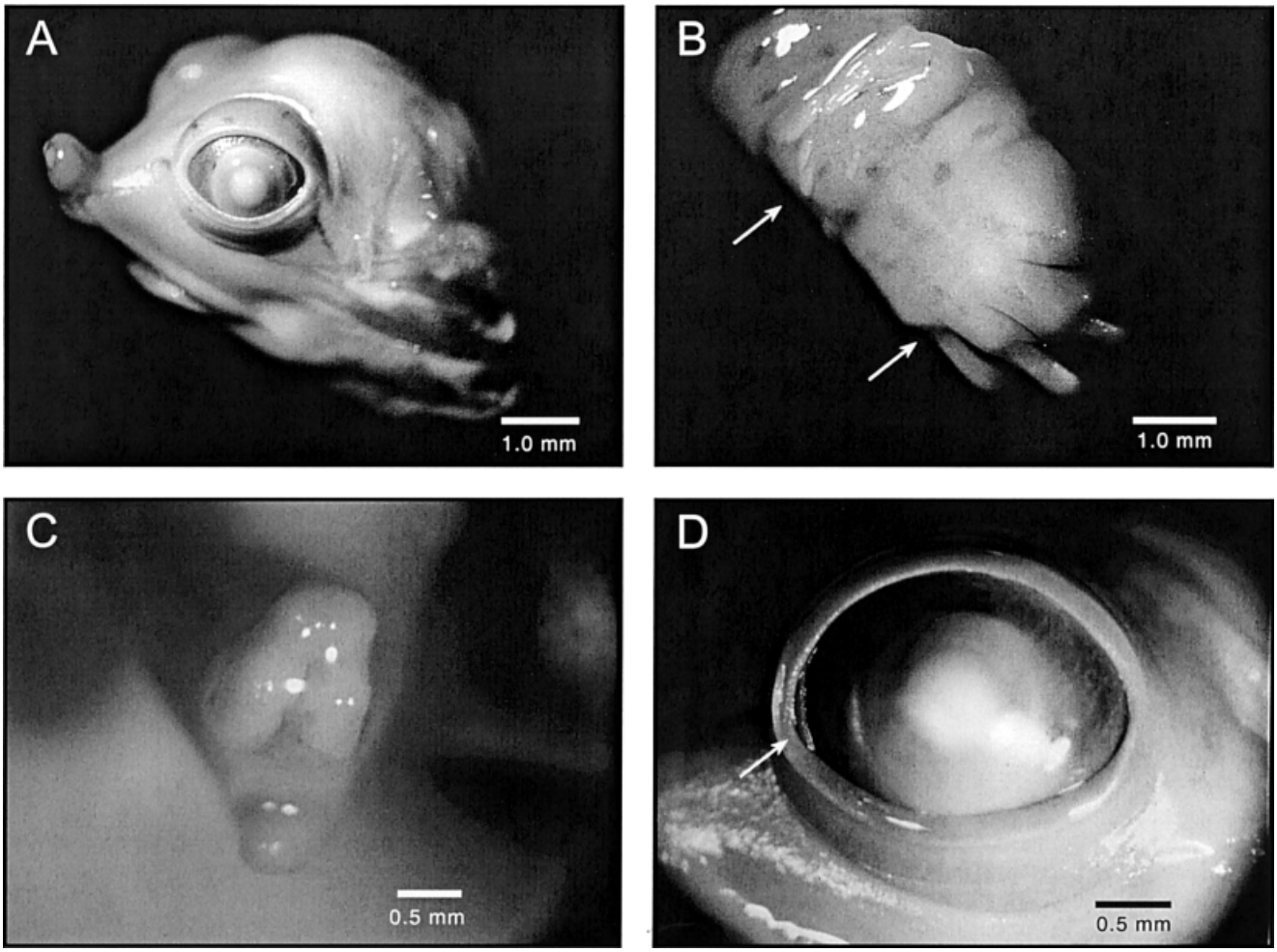

Fig. 6. Photomicrographs of embryonic structures in Stage 20 *Apalone spinifera.* **A:** Lateral view of the head. The anterior margin of the frontal process is absent and the occipital protuberance is greatly reduced; a sharp crease (longitudinal maxillary crease) is forming along the upper edge of the maxillary labium just under the eye. **B:** Forelimb and claw; note the folds on the preaxial–dorsal area of the forelimb are well developed and there is now a sharp crease between the claw and webbing of the forelimb. **C:** Ventral aspect of the urogenital papilla. **D:** Nictitating membrane.

stage cross-references; however, comparison of our Table 3 and their Table 1 will reveal a number of differences. Our equivalencies are explicitly based on the aforementioned morphological features.

Disparate morphological development of different taxa produced comparisons that grouped several stages of a given study into one equivalent Yntema (1968) stage (e.g., Yntema, 1968, Stage 20 is equivalent to Crastz, 1982, Stages 20–25). Moreover, some staging criteria for *Chrysemys picta, Lepidochelys olivacea*, and other sea turtles span several Yntema (1968) stages (e.g., Mahmoud et al., 1973, Stage 22 is equivalent to Yntema, 1968, Stages 23–25). All subsequent comparisons between staging studies in this article refer to Yntema (1968) stages or equivalents derived from comparable morphological features (Table 3).

The first stage at which we have employed a feature not used by Yntema (1968) is Stage 13. The small protuberance between the hindlimbs on the ventral surface at this stage (Fig. 1A) is identical in structure to the cloacal mound described for *Testudo graeca* at Stage 12 (Raynaud and Pieau, 1985). Guyot et al. (1994) described the appearance of a penis anlage as early as Stage 12 in *Testudo hermanni* (Table 4). Crastz (1982) mentioned a genital prominence at Stage 14 of development of *Lepidochelys olivacea*; the structure is not mentioned again in subsequent stages. Renous et al. (1989) noted the appearance of a phallic bud at Stage 14. Although these urogenital structures are homologous, it is misleading to designate them as "inchoate penes" or "phallic" structures because they are present in both sexes. Raynaud and Pieau (1985) did not study the structure in female specimens even though it was noted that adult females possessed a clitoris that was identical in morphological structure to the Stage 26 male embryo phallic anlage. Moreover, Pieau (1974) noted that the phallic anlagen are identical in male and female embryos of the emydid

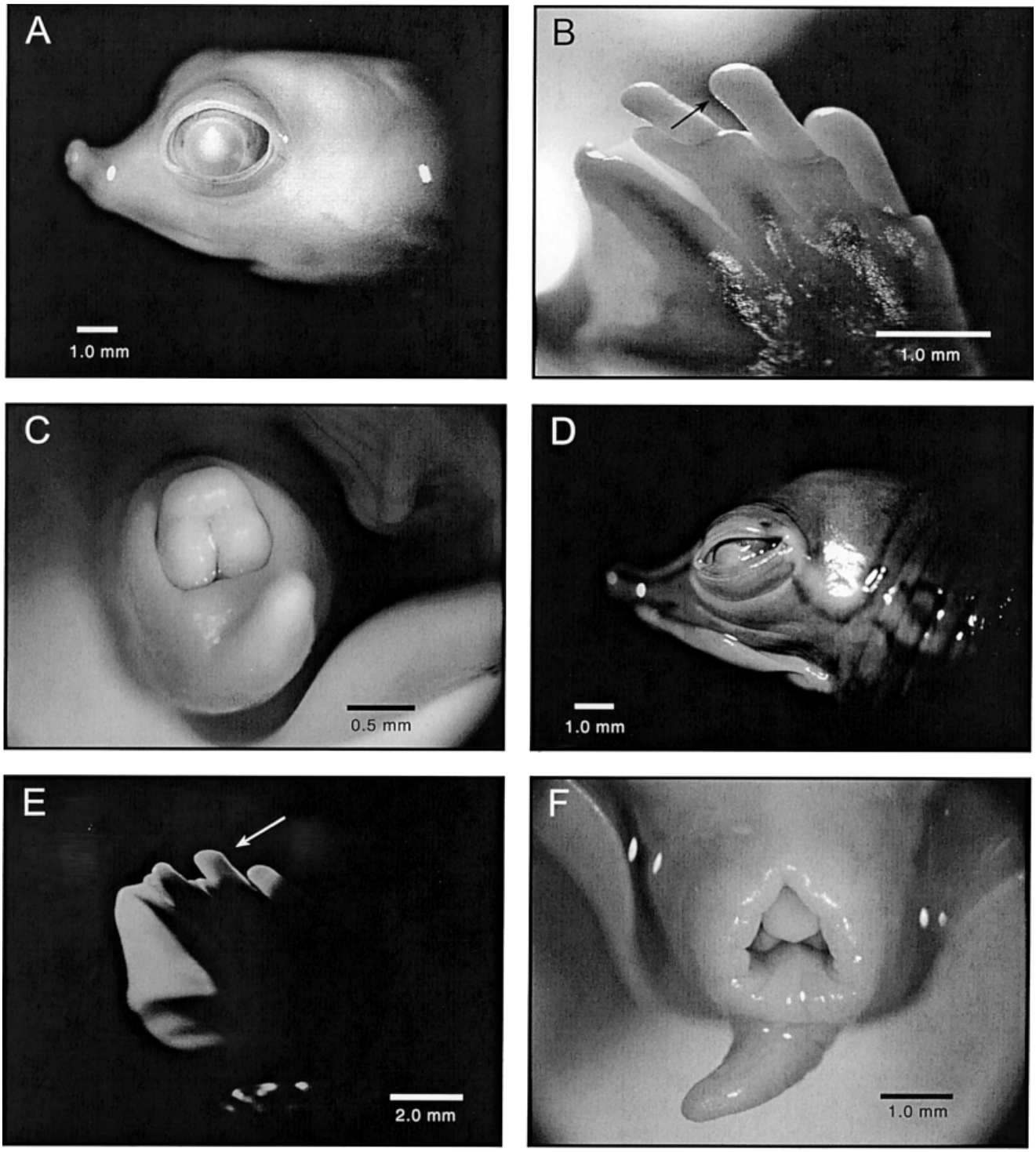

Fig. 7. Photomicrographs of embryonic structures in *Apalone spinifera.* **A:** Lateral view of the head of a Stage 21 embryo. Note that the lower eyelid has crossed the lower margin of the lens; a nictitating membrane can be seen in all individuals of this stage, although it first appears at Stage 20+. **B:** Backlit claw of a Stage 21 embryo; the ungual phalanx can be seen within the claw and the bone has a blunt tip that extends nearly to the tip of the claw. **C:** Ventral aspect of the urogenital papilla of a Stage 21 embryo; note that the structure has begun its descent into the cloaca in this individual. **D:** Lateral view of the head of a Stage 22 embryo; note the conspicuous longitudinal maxillary crease and that the eyelid covers most of the pupil. **E:** Backlit claw of a Stage 22 embryo; although the ungual phalanx extends to a point just proximal to the claw apex, it is tapered in contrast to the blunt bone in the previous stage. **F:** Ventral view of the cloacal orifice of a Stage 22 embryo; the urogenital papilla can be seen within the gaping orifice.

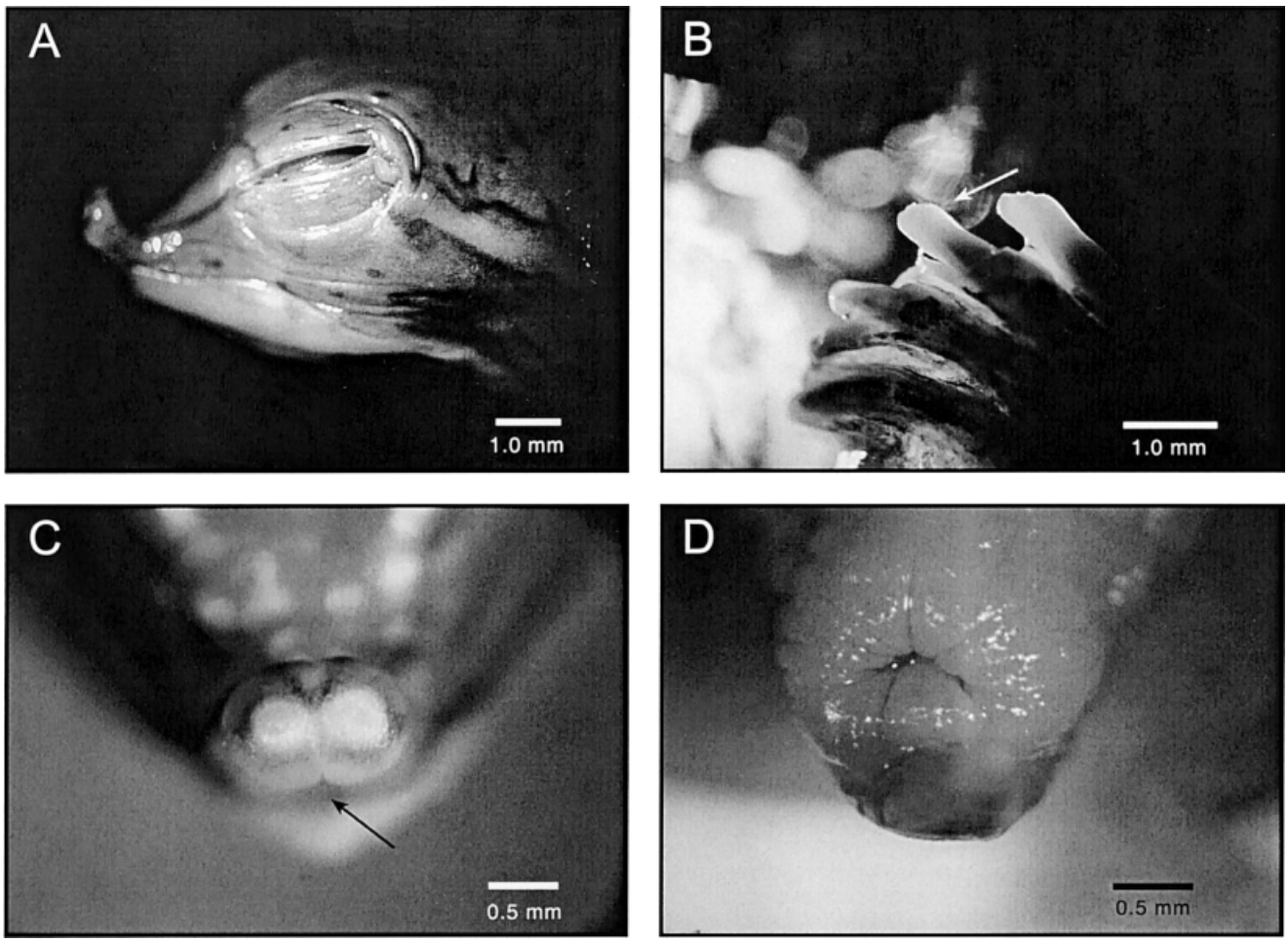

Fig. 8. Photomicrographs of embryonic structures in Stage 23 *Apalone spinifera.* **A:** Lateral view of the head; note that the lower eyelid is separated from the upper by a slit. **B:** Backlit claw; the ungual phalanx begins tapering at a point more proximal to the base of the claw than in the previous stage. **C:** External nares; the narial cavities are sealed and a ventral crease is present. **D:** Ventral view of the vent; the cloaca is sealed shut at the vent with no urogenital papilla visible.

Emys orbicularis. Because sexual differentiation does not occur until about Stage 19 (Wibbels et al., 1994; Greenbaum and Carr, 2001), it is inappropriate to designate the structure as inherently male or female at such an early stage, so we prefer to use the term urogenital papilla to refer to the structure.

At Stage 15 there is a notable difference in the growth of the lower jaw of *Apalone spinifera* relative to that of *Chelydra serpentina* and *Carettochelys insculpta*; the mandible of *A. spinifera* develops earlier than in *Chelydra* or *Carettochelys* (Fig. 1D; Table 4). Mahmoud et al. (1973) noted that the frontal process is evident at Stage 15 in *Chrysemys picta* and that the beak is extended anteriorly at Stage 16, as it is in *A. spinifera*. Also, the appearance of a cloacal crease around the periphery of the urogenital papilla at Stage 15 (Fig. 1E) agrees with Raynaud and Pieau's (1985) description of cloacal membrane resorption at this stage in *Testudo graeca*. Miller (1985) first noted a urogenital papilla at Stage 16 in marine turtle embryos.

At Stage 16, the lower jaw of *Apalone spinifera* extends to the level of the anterior margin of the lens (Fig. 2A), one stage earlier than in the development of *Chelydra serpentina* (Yntema, 1968) and *Carettochelys insculpta* (Beggs et al., 2000; Table 4). Mahmoud et al. (1973) described the development of the lower jaw as it extends anteriorly just under the lens of the eye of *Chrysemys picta* at Stage 17. They also noted that the region of the cloacal orifice in *Chrysemys picta* is distinct and bulges conspicuously at this stage, but whether this description refers to a urogenital papilla is unclear. At Stage 18 of sea turtle development, Miller (1985) noted that the urogenital papilla extends beyond the cloaca. The incipient lips and occipital lobe bifurcation discussed at this stage in *A. spinifera* (Fig. 2A–C) are not reported for *Carettochelys insculpta, Chelydra serpentina, Chrysemys picta, Dermochelys coriacea, Lepidochelys olivacea*, and other sea turtle species, nor *Testudo hermanni* (Beggs et al., 2000; Yntema, 1968; Mahmoud et al., 1973; Renous et al., 1989;

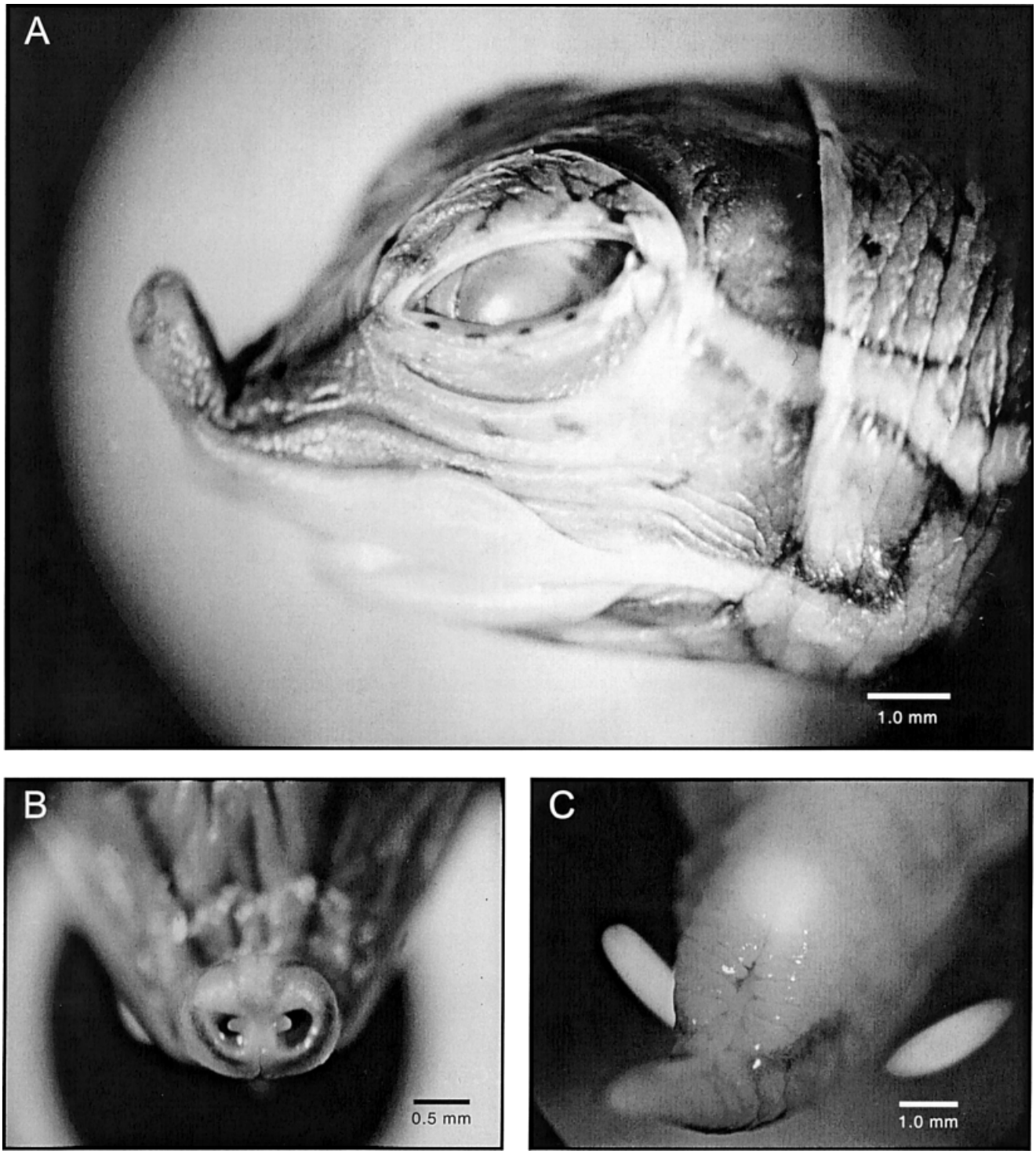

Fig. 9. Photomicrographs of embryonic structures in Stage 24 *Apalone spinifera*. **A:** Lateral view of the head. **B:** Narial cavity; the narial cavities are open, revealing two oval tubercles that project laterally a distance greater than the radius of the narial cavity. **C:** Ventral view of the vent.

Crastz, 1982; Miller, 1985; Guyot et al., 1994, respectively). The possession of maxillary and mandibular labia is unique to members of the family Trionychidae.

Pigmentation of the carapace is first evident at Stage 17 in *Apalone spinifera*; however, in *Chelydra serpentina* dark spots occur as early as Stage 15 on the vertebral scutes (Yntema, 1968). In *Caret-*

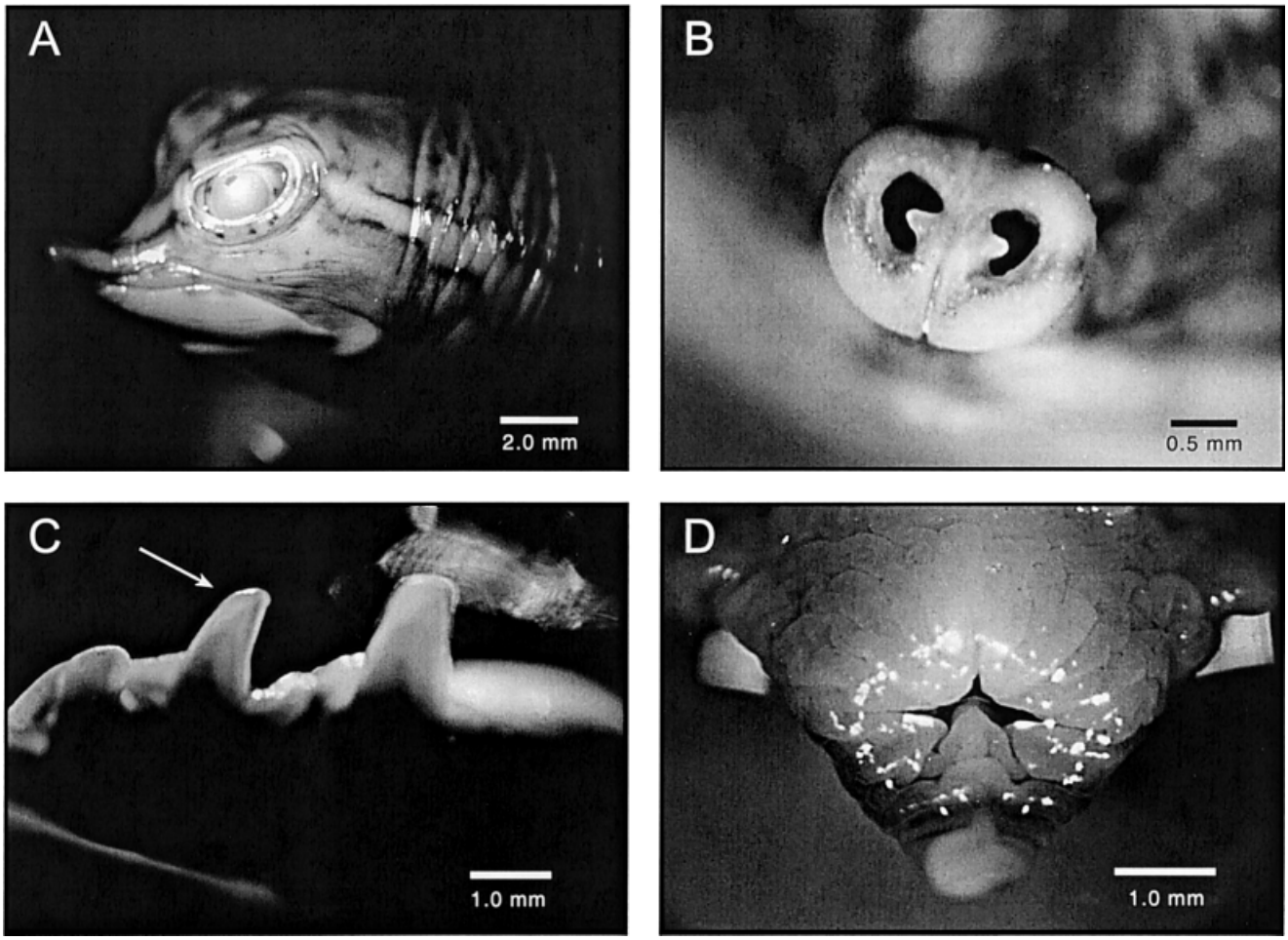

Fig. 10. Photomicrographs of embryonic structures in Stage 25 *Apalone spinifera.* **A:** Lateral view of the head. **B:** Narial cavity; the tubercles project dorsolaterally, are more tapered than in the previous stage, and do not extend beyond the radius of the narial cavity. **C:** Backlit claw; there is slightly more distance between the apex of the ungual phalanx and the apex of the claw. **D:** Ventral view of the vent.

tochelys insculpta carapace pigmentation is first mentioned at Stage 19 (Beggs et al., 2000). Mahmoud et al. (1973) mentioned pigmentation of the carapace beginning at Stage 19 and extending through Stage 26 in *Chrysemys picta*. Pigmentation of the neural plates (= vertebral scutes) is first mentioned at Stage 19 of *Lepidochelys olivacea* (Crastz, 1982). Renous et al. (1989) first note carapace pigmentation for *Dermochelys* at Stage 18, which agrees with its appearance in other sea turtles (Miller, 1985). Carapace pigmentation of *A. spinifera* seems to begin to develop as early as, or earlier than, that of the aforementioned species (Table 4). The near closure of the lower jaw with the upper jaw at Stage 17 of *A. spinifera* (Fig. 3B) is somewhat advanced relative to the condition in *Chelydra, Caretochelys* and *Chrysemys* (lower jaw near the anterior margin of the lens; Yntema, 1968; Beggs et al., 2000; Mahmoud et al., 1973, respectively). Guyot et al. (1994) and Renous et al. (1989) noted that in *Testudo* and *Dermochelys*, respectively, the lower jaw had not progressed anterior to the eye. Guyot et al. (1994) also first illustrated a "penis" in embryonic *T. hermanni* at this stage. In *C. picta*, Mahmoud et al. (1973) mentioned that the "cloacal region" is "distinct" (and again at Stage 19); however, there is no additional explanation, nor an illustration.

The genesis of the caruncle is first noted in *Apalone spinifera* at Stage 18 (Fig. 4C) and Stage 17 in *Chelydra serpentina* and in *Lepidochelys olivacea* at Stage 18 (Yntema, 1968; Crastz, 1982; Table 4). Mahmoud et al. (1973) mentioned the structure at Stage 18, and in *Testudo hermanni* a caruncle bud is noted at Stage 18 (Guyot et al., 1994). The lower jaw has completed development by this stage in *A. spinifera* (Fig. 4B), but it continues to advance anteriorly in *C. serpentina* at this stage and does not terminate development until Stage 19 (Yntema, 1968). In *Chrysemys picta*, it is mentioned that the lower jaw continues to advance through Stage 19, and because this feature is not mentioned again, probably ends its development between Stages 20–21 (Mahmoud

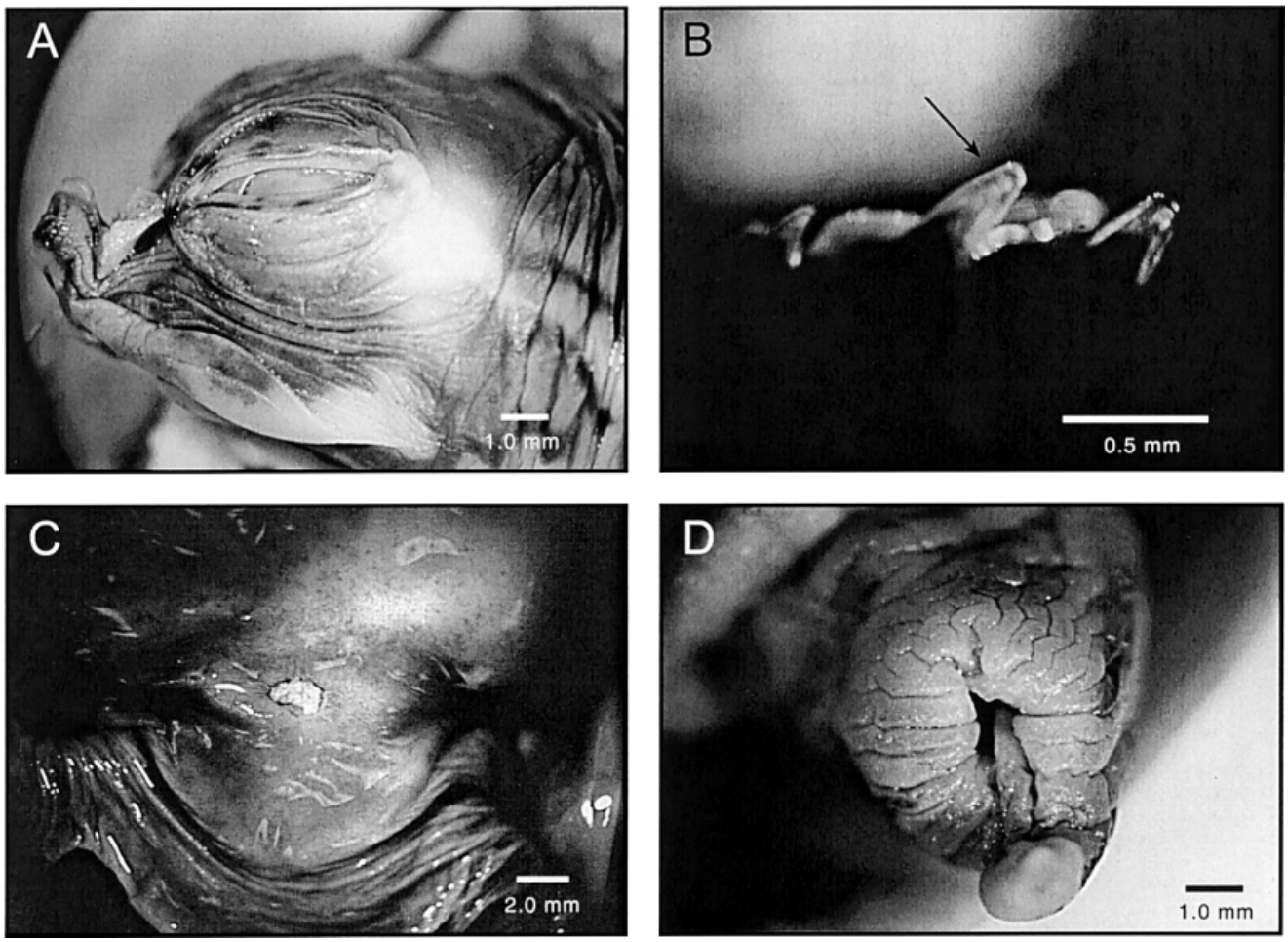

Fig. 11. Photomicrographs of embryonic structures in Stage 26 *Apalone spinifera.* **A:** Lateral view of the head. **B:** Backlit claw; as clearly seen in the middle claw of this individual, the apex of the ungual phalanx is as distant (or further) from the apex of the claw as the width of the claw at the apex of the bone in dorsal view. **C:** Umbilical region; in embryos near hatching such as this individual, the yolk sac is completely internalized, leaving an umbilical scar. **D:** Ventral view of the vent.

et al., 1973). Complete closure of the jaws is noted in *Lepidochelys olivacea* at Stage 20, slightly later than the above species (Crastz, 1982). A "penis" is illustrated at Stage 18 of *T. hermanni*, noted as still obvious at Stage 20, and then not shown again (Guyot et al., 1994).

The digit-webbing differentiation first observed during Stage 19 in *Apalone spinifera* (Fig. 5D) is a useful characteristic because the Yntema (1968) criterion of digit length is a more subtle and subjective distinction between Stages 19 and 20; however, the combination of these two digital features, used in conjunction with eyelid morphology, can ensure confident staging of embryos (Fig. 5A). Yntema (1968) mentioned that the claws are distinct from the digits at the web at Stage 21; although the significance of this is unclear, it is considerably later than the differentiation of these structures noted for *A. spinifera*.

The results of backlighting the digits and claws is first used as a criterion at Stage 20 in *Apalone spinifera*. Although Yntema (1968) first began to explain claw morphology at Stage 23 of *Chelydra serpentina* development, his descriptions of characteristics are somewhat confusing and lack unequivocal elaboration or illustration. Therefore, the changes in ungual phalanx and claw morphology as described for *A. spinifera* are novel and represent useful staging criteria in conjunction with other morphological features. The preaxial–dorsal folds of the forelimb are obvious at Stage 20 (Fig. 6B), but do not become apparent in *C. serpentina* until Stage 21 (Yntema, 1968). The concealment of digits IV and V at Stage 20 is unique to *Apalone* (and other trionychids) because of the postnatal presence of only three clawed digits per limb (Fig. 6B). Although the urogenital papilla of specimens at Stage 20 projects through the vent (Fig. 6C), Miller (1985) noted that the structure was withdrawn into the cloaca of sea turtles at Stage 21. It is unclear whether the cloaca is completely sealed or whether the urogenital papilla is

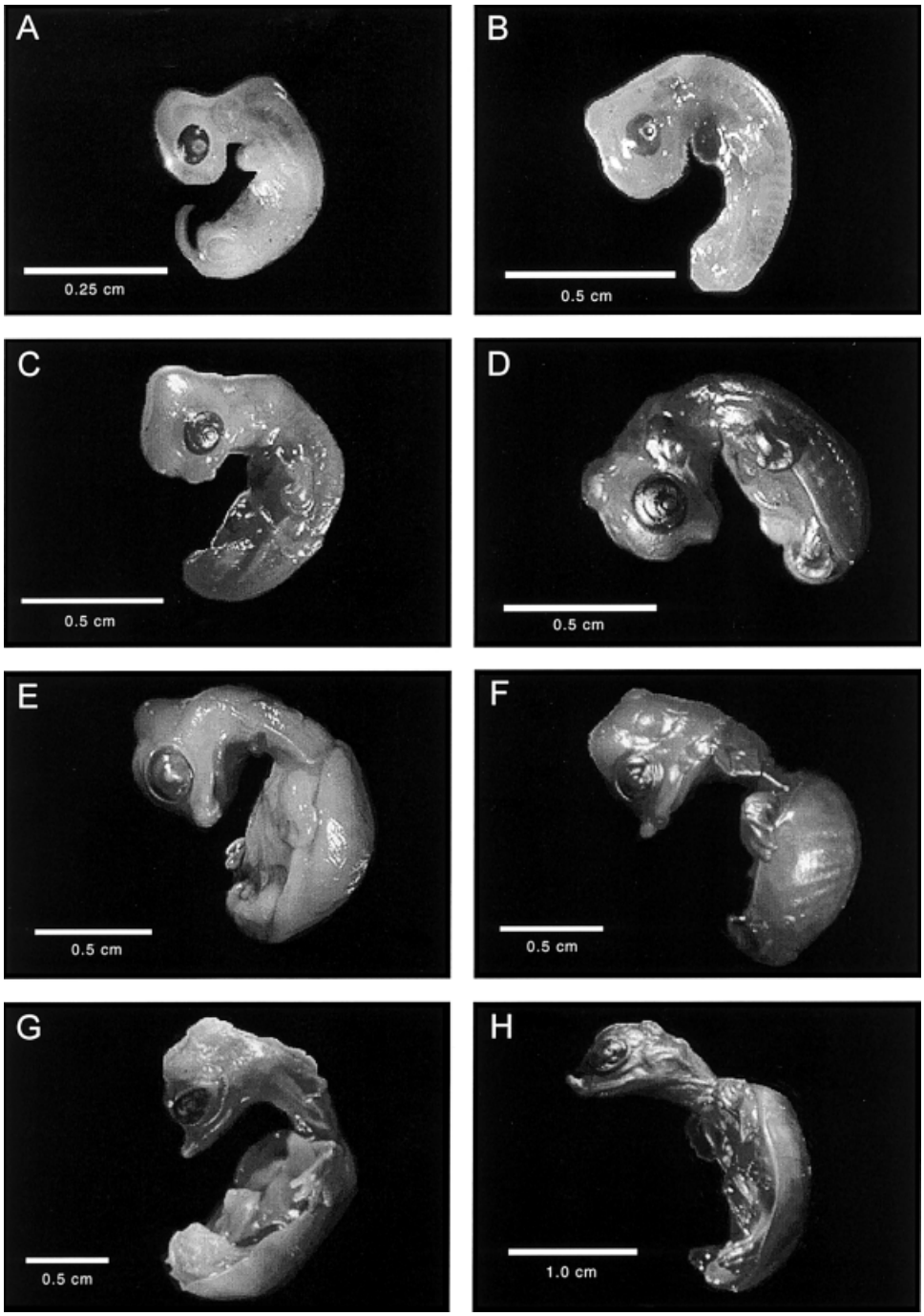

Fig. 12. Photomicrographs of whole embryos in lateral view. **A:** Stage 13. **B:** Stage 14. **C:** Stage 15. **D:** Stage 16. **E:** Stage 17. **F:** Stage 18. **G:** Stage 19. **H:** Stage 20.

visible within the gaping vent in the sea turtles. Use of the nictitating membrane as a developmental criterion is novel; in *A. spinifera* it is present in Stage 20+ and in all individuals at Stage 21 (Figs. 6D, 7A). Guyot et al. (1994) did not mention the structure but it is clearly visible in illustrations of Stage 21 in *Testudo hermanni* and may be present at Stage 20.

The longitudinal maxillary crease characteristic described for Stage 21 is also novel. Although highly variable within this stage, the subtle distinctions of the labia between stages in conjunction with other characteristics confirm precise developmental stage of all individuals. Because the urogenital papilla ranges from fully extended to completely withdrawn and sealed inside the cloaca, there is no correlation between sex and position of the structure (Fig. 7C). Mahmoud et al. (1973) noted that the bulge of the cloacal region is inconspicuous at Stages 21–22, but no illustration or detailed explanation is given to allow comparison. Scleral papillae disappear at Stage 21 in *A. spinifera* and *Carettochelys* (Beggs et

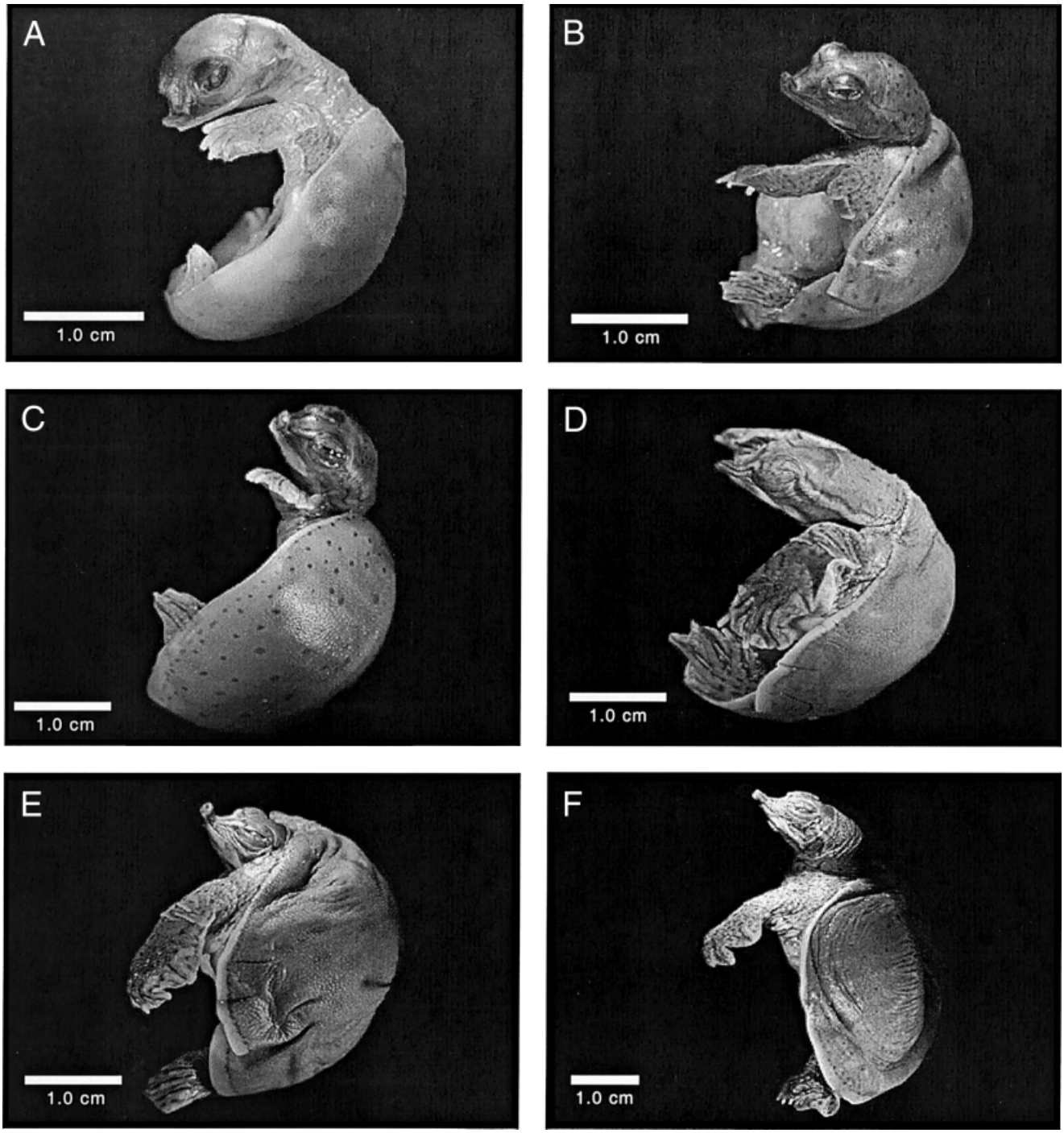

Fig. 13. Photomicrographs of whole embryos in lateral view. **A:** Stage 21. **B:** Stage 22. **C:** Stage 23. **D:** Stage 24. **E:** Stage 25. **F:** Stage 26.

al., 2000), while Yntema (1968) noted their presence as late as Stage 20 in *Chelydra serpentina,* and Mahmoud et al. (1973) reported their disappearance at Stage 20.

In *Apalone spinifera*, the urogenital papilla has begun to retreat into the cloaca in all individuals by Stage 22 and is concealed completely by Stage 23 (Figs. 7F, 8D). Risley (1933) noted that in all individuals of *Sternotherus odoratus* between 11.0 and 14.0 mm in carapace length, the "phallus" was withdrawn into the cloaca. Because sexual differentiation has not yet occurred at this embryo length, we estimate that the embryos are Yntema (1968) Stage 17–18. This pattern of urogenital papilla development differs markedly from that observed in *A. spinifera* and other turtles for which the phenome-

TABLE 1. Duration of Yntema (1968) stages in Apalone spinifera *at 26°C and 31°C*

Stage	Temperature	
	26°C	31°C
11	4.15 (2) ± 0.05	—
12	3.96 (5) ± 0.73	—
13	4.13 (10) ± 1.19	—
14	4.19 (12) ± 1.26	2.35 (2) ± 0.21
15	4.68 (13) ± 1.23	2.51 (7) ± 0.67
16	4.97 (15) ± 1.11	3.61 (10) ± 3.35
17	5.19 (13) ± 0.96	3.69 (12) ± 3.05
18	4.92 (12) ± 0.80	2.90 (12) ± 0.92
19	5.05 (12) ± 1.04	3.36 (13) ± 1.60
20	6.15 (11) ± 3.95	4.32 (12) ± 1.99
21	6.71 (10) ± 4.04	5.48 (14) ± 2.49
22	6.01 (6) ± 2.06	6.30 (11) ± 2.26
23	6.38 (5) ± 2.06	5.00 (6) ± 1.02
24	6.38 (5) ± 2.06	4.69 (4) ± 1.13
25	6.38 (5) ± 2.06	4.69 (4) ± 1.13
26	7.40 (1)	4.69 (4) ± 1.13

Data are average number of days per stage (sample size) ± 1 SD.

TABLE 3. Equivalent stages of turtle development

Yntema (1968)	Miller (1985)*	Crastz (1982)	Mahmoud et al. (1973)
13	17	11	12
14	18–19	12	13
15	20–21	13–14	14–15
16	22	15	16
17	23	16	17
18	24–25	17–18	18
19	26	19	19
20	27	20–25	20
21	28	26–28	21
22	28	26–28	21
23	29	29	22
24	29	29	22
25	30	30	22
26	31	31	23

* Renous et al. (1989) and Billett et al. (1992) stages are equivalent to those of Miller (1985).

non has been described (Raynaud and Pieau, 1985). From Stage 24 on, none of Yntema's (1968) criteria can be used to stage *A. spinifera* embryos; however, our staging criteria are based on comparable morphological features that parallel the development of *Chelydra serpentina* for Stages 24–26 (Figs. 9–11).

Comparison to Regression-Line Method of Staging

The Australian turtle *Carettochelys insculpta* (Carettochelyidae) is usually regarded as the sister group to the Trionychidae (Gaffney, 1984; Iverson, 1992, and citations therein). Prior to the study of Beggs et al. (2000), Webb et al. (1986) used development rate – temperature coefficients and head width – egg shell diameter ratios to predict development rates of this species. Regression equations were used to compare equivalent Yntema (1968) stages to the approximate ages of the *Carettochelys* embryos in order to stage them. This methodology was derived from procedures used to estimate the age of crocodilians collected in wild populations (Webb et al., 1983). The method was neither comparable to any previous studies of turtle embryology nor did it apply beyond Stage 24 because a plateau in head width was noted for *C. insculpta* from Stages 24–26 (Webb et al., 1986).

It was not possible to use this method to stage embryos of *Apalone spinifera* for several reasons. The method for staging *Carettochelys insculpta* assumes that head width growth will increase linearly and that duration between stages will be relatively constant. In *A. spinifera* raised at 26°C, average head width remained constant between Stages 18 and 19 and Stages 25 and 26 (Table 2). Similarly, head width decreased or remained constant between

TABLE 2. Measurements of embryos of A. spinifera *for Yntema Stages 11–26 at two different temperatures*

Stage	26°C		31°C		Total	
	HW	CL	HW	CL	HW	CL
11	1.3 (2) ± 0.18	—	—	—	1.3 (2) ± 0.18	—
12	1.9 (3) ± 0.18	—	1.2 (1)	—	1.7 (4) ± 0.34	—
13	2.2 (4) ± 0.11	—	—	—	2.2 (4) ± 0.11	—
14	2.8 (2) ± 0.54	—	2.5 (2) ± 0.26	—	2.7 (4) ± 0.59	—
15	3.5 (3) ± 0.26	—	3.3 (4) ± 0.26	—	3.4 (7) ± 0.27	—
16	4.6 (4) ± 0.66	8.3 (4) ± 0.93	4.4 (4) ± 0.04	7.3 (4) ± 0.39	4.5 (8) ± 0.45	7.8 (8) ± 0.87
17	4.3 (2) ± 1.80	7.7 (2) ± 0.16	4.5 (5) ± 0.35	8.7 (5) ± 0.56	4.5 (7) ± 0.57	8.4 (7) ± 0.95
18	5.4 (2) ± 0.26	12.6 (2) ± 0.13	5.4 (4) ± 2.30	9.2 (4) ± 0.02	5.4 (6) ± 1.80	10.3 (6) ± 2.10
19	5.4 (5) ± 0.37	14.1 (5) ± 0.78	5.4 (4) ± 0.14	14.6 (4) ± 1.16	5.4 (9) ± 0.18	14.4 (9) ± 0.88
20	5.7 (4) ± 0.14	16.5 (4) ± 0.16	5.2 (3) ± 0.21	14.6 (3) ± 1.19	5.5 (7) ± 0.27	15.7 (7) ± 2.00
21	5.9 (8) ± 0.45	20.4 (8) ± 2.30	5.4 (15) ± 0.54	18.7 (15) ± 1.90	5.6 (23) ± 0.56	19.3 (23) ± 2.15
22	6.8 (1)	25.6 (1)	6.0 (5) ± 0.57	21.8 (5) ± 1.90	6.1 (6) ± 0.61	19.3 (6) ± 4.14
23	—	—	6.4 (5) ± 0.86	25.5 (5) ± 1.50	6.4 (5) ± 0.86	25.5 (5) ± 1.50
24	6.3 (1)	23.9 (1)	7.0 (4) ± 0.53	26.1 (4) ± 1.25	6.9 (5) ± 0.40	25.6 (5) ± 1.50
25	7.8 (4) ± 0.33	30.8 (4) ± 2.23	7.8 (4) ± 0.19	34.3 (4) ± 1.06	7.8 (8) ± 0.25	32.6 (8) ± 2.50
26	7.8 (2) ± 0.46	28.7 (2) ± 3.56	7.3 (5) ± 0.43	32.3 (6) ± 4.50	7.5 (7) ± 0.45	31.0 (8) ± 4.20

Data are average lengths in mm (sample size) ± 1 SD, HW = head width, CL = carapace length.

TABLE 4. Comparison of selected staging criteria for embryonic turtles

Character and stage	*Apalone* This study	*Carettochelys* Beggs et al. (2000)	*Testudo* Guyot et al. (1994)	*Dermochelys* Renous et al. (1989)	Sea turtles Miller (1985)	*Lepidochelys* Crastz (1982)	*Chrysemys* Mahmoud et al. (1973)	*Chelydra* Yntema (1968)
Urogenital papilla		Not documented						Not documented
Stage 12	—	—	Penis anlage noted	—	—	—	—	—
13	First evident as small protuberance	—	—	—	—	—	—	—
14	—	—	Penis bud noted	Phallic bud evident	—	Genital prominence evident	—	—
15	Vent crease forms around papilla	—	—	—	—	—	—	—
16	Protrudes from vent	—	—	Prominent	First evident	—	—	—
17	—	—	Distinct	—	—	—	Cloacal region distinct	—
18	—	—	—	Partly covered by cloaca	Protrudes from cloaca	—	—	—
19	—	—	—	—	—	—	Noted as distinct (again)	—
20	Still protrudes from vent	—	Still obvious	Mostly covered by the cloaca	—	—	—	—
21	—	—	—	—	Withdrawn into cloaca	—	Structure inconspicuous	—
22	—	—	—	—	—	—	—	—
23	Withdrawn into cloaca and vent closed	—	—	—	—	—	—	—
Lower jaw								
Stage 15	Reaches anterior edge of pupil	Extends to posterior edge of eye	—	—	—	—	—	Reaches posterior edge of eye
16	Reaches anterior margin of lens	Encroaches on posterior edge of lens	Reaches posterior border of lens	Reaches posterior border of eye	—	—	—	Encroaches on posterior edge of lens
17	—	—	Reaches anterior margin of eye	Reaches anterior border of eye	—	—	—	Reaches just past anterior edge of lens
18	Complete closure with upper jaw	Reaches frontonasal groove	Mouth is nearly complete	—	Slightly posterior to upper jaw length	Reaches anterior border of eye	—	—
19	—	—	—	Mouth almost has reached complete closure	—	—	Extends between eye and frontal process	Development complete
20	—	—	—	—	—	Complete closure with upper jaw	Development complete?	—
Carapace pigmentation								
Stage 15	—	—	—	—	—	—	—	First evident
16	—	—	—	—	—	—	—	—
17	First evident	—	—	—	—	—	—	—
18	—	—	—	First evident	First evident	—	—	—
19	—	First evident	—	—	—	First evident	First evident	—
20	—	—	First evident	—	—	—	—	—
Caruncle		Not documented						
Stage 17	—	—	—	First evident	—	—		First evident
18	First evident	—	First evident	—	First evident	First evident	First evident	—

Stages 18 and 21 in embryos developing at 31°C and decreased between Stages 25 and 26 (Table 2). Application of the method of Webb et al. (1983) would have produced erroneous staging for *A. spinifera*, given the widely variable duration of stages in its development (see Table 1). As previously reported for *Chelydra serpentina* (Yntema, 1978), during the later stages of development there was much less difference in the rate of development at different temperatures than there had been during early development. This sort of heterogeneity in developmental rates may account for our observations on head width dimensions and the duration of stages at the two temperatures in *Apalone* (i.e., declines from one stage to the next and plateaus).

Moreover, prediction of developmental stages with morphometric data provides little useful information and has limited applicability to other populations. Because dwarfism and gigantism are known to occur among closely related populations of Australian reptiles (O'Shea, 1991), it is likely that the staging equation used for one population may not be effective for another if the embryos are significantly different in size, as having been incubated at different temperatures (see Table 2). For example, a formula used to stage *Apalone spinifera* would not work for a population of *A. mutica* because the latter species is much smaller. The vitelline sac diameter data Crastz (1982) used to stage embryos (Crastz Stages 30 and 31) are equally limited in their application to other taxa, as well as other conspecifics incubated at disparate temperatures.

Qualitative treatment of morphological features can be applied to describe development in diverse taxa and doubtless will yield more productive comparisons than using quantitative data such as regression equations. Smith (2001) noted how comparisons based on external measurements are not useful for the sort of standardization of developmental phenomena that can lead to the study of heterochrony. However, Renous et al. (1989) noted that previous workers differed in their description of morphological features used for turtle staging criteria. Moreover, the use of staging schemes with different numbers of stages is an impediment to interspecific comparisons. Once basic staging criteria have been described for all chelonian families, a universal staging scheme can be constructed to facilitate embryonic comparisons of all turtles in a fashion similar to the Gosner (1960) stages of larval anurans.

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A recent paper on the embryology of *Pelodiscus sinensis* (Tokita and Kurantani, 2001) was brought to our attention after this paper was in press. It should be consulted for potentially useful information to compare with *Apolone spinifera*.

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