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Sexual Differentiation in the Spiny Softshell Turtle (*Apalone spinifera*), a Species With Genetic Sex Determination

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ABSTRACT It is hypothesized on the basis of sex determination theory that species exhibiting genetic sex determination (GSD) may undergo sexual differentiation earlier in development than species with environmental sex determination (ESD). Most turtle species exhibit a form of ESD known as temperature-dependent sex determination (TSD), and in such species the chronology of sex differentiation is well studied. *Apalone spinifera* is a species of softshell turtle (Trionychidae) that exhibits GSD. We studied sexual differentiation in this species in order to facilitate comparison to TSD species. Eggs were incubated at two different temperatures and embryos were harvested at various stages of mid to late development. Gonad length was measured with image analysis software, then prepared histologically. Indifferent gonads have differentiated in stage 19 embryos. Histological details of gonadogenesis follow the same pattern as described for other reptiles. Regression of the male paramesonephric duct closely follows testicular differentiation. Gonad lengths are longer at the warmer incubation temperature, and ovaries are generally longer than testes at each stage and for each temperature. Although sexual differentiation takes place at about the same stage as in other turtles with TSD (18–20), in *A. spinifera* this differentiation is irreversible at this stage, while in some of the TSD species sex is reversible until about stage 22. This immutable, definitive sexual differentiation may support the hypothesis of an accelerated chronology of sex differentiation for this species. We also note that sexual dichromatism at hatching is known in this species and may provide additional evidence of early differentiation. *J. Exp. Zool. 290:190–200, 2001.*

Sexual differentiation and sex determination have been the subject of increasing research interest in nonmammalian vertebrates, especially reptiles (see recent reviews in Pieau, '96; Lance, '97; Lance and Bogart, '98). Lance ('97) distinguishes between the two related phenomena, defining sexual differentiation as "a programmed cascade of genetic and hormonal events in which the indifferent gonad develops as a testis or ovary with the appropriate urogenital and secondary sex characters," and sex determination as the event or trigger that sets off the cascade. Given the diversity of sex determining mechanisms known among reptiles (Ewert and Nelson, '91; Ewert et al., '94; Wibbels et al., '94), it is surprising that essentially all studies of sexual differentiation have involved species that exhibit temperature-dependent sex determination (TSD) (Lance, '97). Among turtles, TSD is very common and has been found in most turtle families, while genetic (or genotypic) sex determination (GSD) has been found in relatively few taxa.

Studies involving GSD in turtles are limited in number and scope, having primarily consisted of documenting that primary sexual differentiation at the time of hatching is not influenced by temperature, including some species that have sex chromosomes (see review in Janzen and Paukstis, '91). Other species that lack heteromorphic chromosomes nonetheless have been shown to have GSD, including several chelids, an emydid, and two species of the Trionychidae (Bull and Vogt, '79; Yntema, '81; Vogt and Bull, '82; Bull et al.,

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'85; McBee et al., '85; Georges, '88; Thompson, '88; Ewert and Nelson, '91). Although it exhibits GSD, the spiny softshell turtle (*Apalone spinifera*) lacks heteromorphic sex chromosomes (Bickham et al., '83). Embryos of *A. spinifera* have been reported to be susceptible to feminization by administration of exogenous estradiol (Bull et al., '88); however, a more recent study found no influence of sex steroids on sex ratio in this species (Marquez et al., '99).

Researchers have documented the morphological chronology of primary sexual differentiation in terms of developmental stages; however, in all cases the species involved exhibit TSD (Yntema, '79; Bull and Vogt, '81; Pieau and Dorizzi, '81; Yntema and Mrosovsky, '82; Wibbels et al., '91a). Broadly speaking, the thermosensitive period (TSP) extends between stages 14–22 and is very comparable across numerous taxa. These stages correspond with a time that begins while the gonad is sexually indifferent and continues through a point when sex-specific gonadal differentiation has begun (Wibbels et al., '94). This is roughly the same developmental time frame during which the embryos are susceptible to hormonal manipulation of gonadogenesis (Pieau, '96; Lance, '97). In every case, primary sexual differentiation of the gonads had taken place by the time of hatching (stage 26), with the exception of a few intersexes with ovotestes found in some species (reviewed in Pieau et al., '98). Another outcome of the temperature sensitivity experiments has been the observation that there is a differential effect of temperature on the growth and development of ovaries in *Trachemys scripta* and *Graptemys caglei* (Wibbels et al., '91a,b).

Charnov and Bull ('77) formulated a general model concerning the evolution of ESD. Part of the rationale relates the timing of ultimate sexual differentiation to mode of sex determination. Their hypothesis suggests that when environmental factors determine the sex of an embryo as in species with TSD, it would be advantageous to postpone sexual differentiation as long as possible in order that the developing embryo may experience and respond to the environmental cue that will give rise to the sex having the greatest "relative" fitness. Therefore, in GSD species, since there is no environmental influence, sexual differentiation may begin immediately, resulting in the early development of sex, which may be of significant fitness advantage to such individuals and this may be the "major advantage of GSD" (Charnov and Bull, '77). Since embryonic gonadogenesis results in primary sexual differentiation prior to hatching or birth in amniotes, according to the Charnov-Bull model, it seems logical to predict a heterochronic change in embryonic sexual differentiation between ESD and GSD species.

The goal of this study is to test the hypothesis that a GSD species of turtle, *Apalone spinifera*, will undergo primary gonadal differentiation at an earlier stage of embryonic development when compared to TSD species, such as *T. scripta* (Wibbels et al., '91a). In addition, gonadal development of *A. spinifera* will be compared at two different temperatures to test for the presence of temperature effects other than on sex, as have been reported on ovarian length (Wibbels et al., '91a,b).

MATERIALS AND METHODS

Egg collection and incubation

A total of 306 eggs of *Apalone spinifera* were obtained for experimentation in 1996 and 1997. Forty-four eggs collected in West Feliciana Parish, Louisiana in the second half of May, 1996, plus 46 eggs obtained in Ouachita Parish were maintained at a room temperature near 24.5°C until June 10, 1996. At that time the eggs were all moved to an incubator set at 31°C (Precision Scientific Lab Incubator). Another 36 eggs obtained in Ouachita Parish, Louisiana in May and June, 1997 were also incubated at 31°C. One hundred eighty eggs obtained on July 4, 1997 from Concordia Turtle Farm in Wildsville, Louisiana were randomly divided and placed into incubators on July 9 at 31°C or July 10 at 26°C, the same two temperatures employed by Bull et al. ('88) and Wibbels et al. ('91a). In 1997, eggs were placed into insulation board incubators ($60 \times 70 \times 74$ cm) with the temperature regulated by a digital thermostat (Lang et al., '89). Eggs were individually numbered and placed in small plastic boxes before being placed in an incubator. The incubation substrate used in the boxes contained a 1:1 mixture of granular vermiculite to distilled water by weight. During the course of the experiment, the temperature of the incubators fluctuated as much as 0.6°C around the desired setpoint in 1997 and 1.0°C in 1996.

Embryo collection

Embryos incubated in 1996 were sacrificed starting on 26 June 1996 by severing the spinal cord. Embryos from 1997 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 pentabarbitol if they had a to-

tal length exceeding 1 cm (AVMA Panel on Euthanasia, '93). Embryos from 1996 were preserved in 10% formalin for 12 months before transferral to Bouin's solution for at least two weeks, and then stored in 50% isopropyl alcohol (Presnell and Schreibman, '97). Embryos from 1997 were directly preserved in Bouin's solution for at least two weeks before transferral to 50% isopropyl alcohol for longer term storage. A total of 32 embryos were harvested from the 1996 clutches, and 101 embryos were collected from the 1997 eggs. Research on turtle embryos was approved by the Institutional Animal Care and Use Committee.

Histology

Yntema ('68) described 26 stages in the embryonic development of *Chelydra serpentina*, and most studies of turtle embryos since then have used his stages as the standard by which to compare the timing of developmental events. Each embryo was assigned to the appropriate embryonic stage using the morphological criteria of Yntema ('68), supplemented by characteristics specific to *A. spinifera* (Greenbaum and Carr, personal communication). Whole embryos from stages 11–14 and the kidney + gonad from embryos staged 15–26 were cut transversely, transferred to individually labeled cassettes, and immersed in a tissue processor for 12 hr. The two tissue pieces from each embryo were positioned with their cut ends down against the embedding plates and covered in paraffin (Yntema, '81). Every tenth section was mounted and stained using hematoxylin-eosin stains as outlined by Presnell and Schreibman ('97), with the following exceptions: the absolute alcohol bath (Step 2), 70% alcohol bath (Step 4), Lugol solution bath (Step 5), 5% thiosulfate bath (Step 7), and the optional absolute alcohol-xylene bath (Step 18) were skipped. The Scott solution bath (Step 11) was substituted with a 1% HCl bath, and a 1% ammonium hydroxide bath was employed directly following the water bath (Step 12). Eosin was the counterstain used, and the coverslips were mounted with Cytoseal (Stephens Scientific, Riverdale, NJ).

Whole preserved gonads were photographed through a stereo microscope using an Industrial Color CCD video camera (Panasonic Broadcast and Television Systems Company, Secaucus, NJ). The image was captured to a computer file using Snappy Video Snapshot software (Play Incorporated, Rancho Cordova, CA) and then measured with Sigma Scan Pro Image Measurement software (Jandel Corporation, San Rafael, CA). All statistics on measurements and frequencies were executed with The SAS System for Windows, release 6.12. Statistics employed included chi-square analysis to test frequencies, and student's *t*-test for means.

RESULTS

Histological description of gonads and paramesonephric duct

The following description is based upon examination of histological sections of the kidney and gonad complex of embryos from stages 15 to 26. Chi-square analysis of the total sex ratio for all sexually diagnosable embryos based on histology, indicated that it was not different from a 1:1 ratio ($\chi^2 = 0.134$, n = 67, $P = 0.714$). A 2×2 contingency table of sex and incubation temperature indicated that the two factors are independent of one another ($\chi^2 = 0.070$, n = 67, $P = 0.792$). These data are in accord with prior studies which concluded that *A. spinifera* has GSD rather than TSD (Bull and Vogt, '79; Yntema, '81; Vogt and Bull, '82).

Stage 15 gonads lack distinctive internal structure. Germ cells are present in the gonad, which projects outward slightly from the kidney.

At stage 16 the cortical layer of the gonad is 1–2 cells thick, but does not have a distinct boundary separating it from the medullary area. The cortex is darkly stained at its periphery. Sex cords are present, but indistinct, with nuclei that stain more darkly than those in other cells of the gonad. Germ cells are scattered throughout the gonad and can be observed in mitotic division. There remains an extensive attachment of gonad to kidney. The paramesonephric duct bulges as a small ridge on the side of the kidney, and may vary from a nearly circular array of cells with darkly-stained nuclei in the center, to possessing a discrete ring of simple cuboidal or columnar epithelium surrounding a narrow lumen.

The stage 17 gonad remains indifferent and lacking in significant internal organization as was exhibited in the prior stage (Fig. 1A). Paramesonephric ducts are still growing at this stage, and depending on where the transverse section is made, display variation in the structure of the epithelial lining. Stromal mesenchyme cells surround the epithelial ring in all ducts, and about half the circumference of the wall of the structure is still connected to the kidney (Fig. 1B).

By stage 18, the gonad has developed a relatively less extensive contact with the kidney and has cells with dark staining nuclei within the sex cords, some of which are now easily discernible.

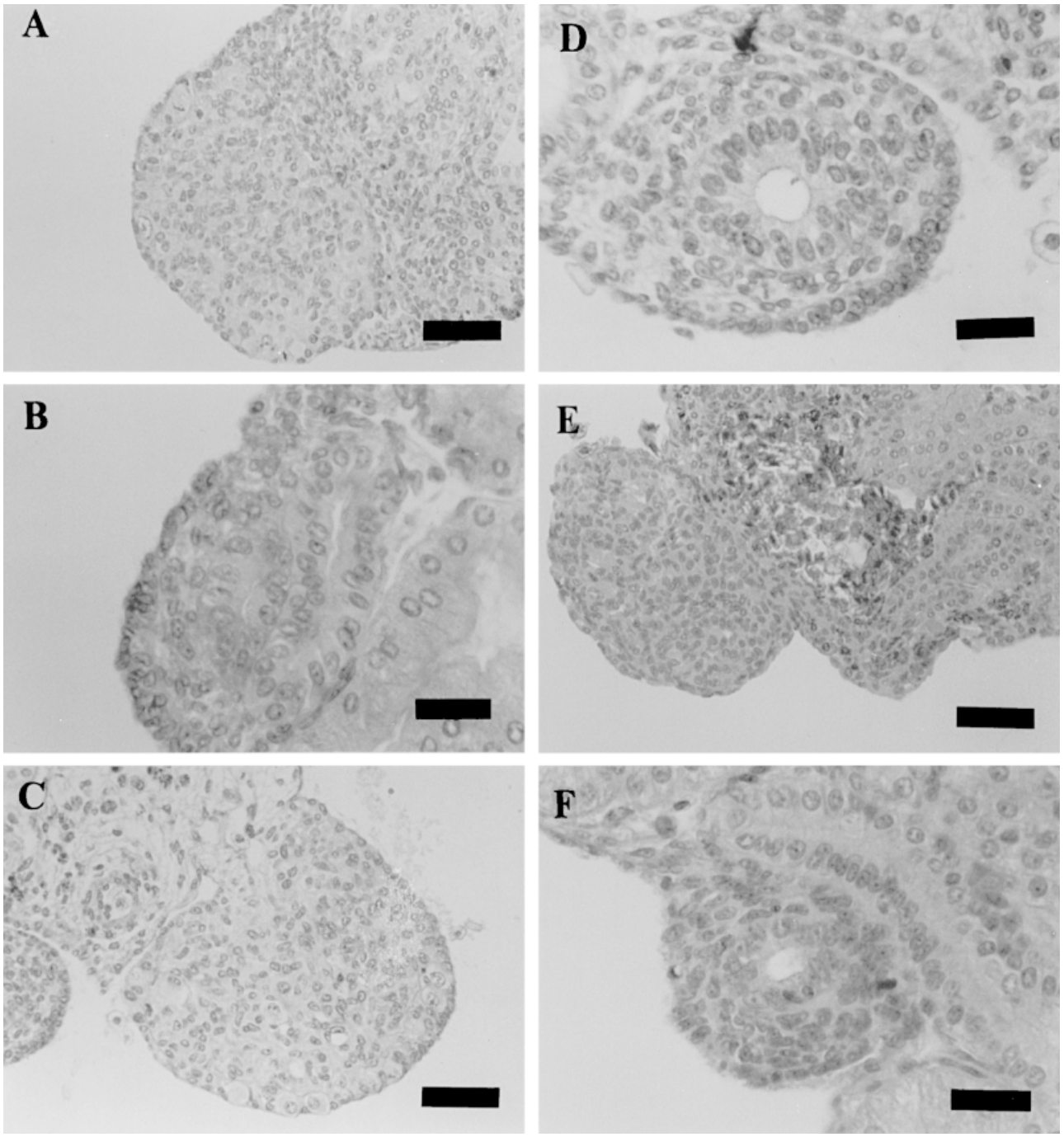

Fig. 1. (**A**) Stage 17 gonad. Scale bar = 50 μm. (**B**) Stage 17 paramesonephric duct. Scale bar = 25 μm. (**C**) Stage 19 female gonad. Indications of sex cords are absent and the cortex region is prominent. Scale bar = 50 μm. (**D**) Stage 19 female paramesonephric duct. Nuclei in the epithelial ring of cells are at the basal side. The structure is partially detached from the kidney. Scale bar = 25 μm. (**E**) Stage 19 male gonad. Indications of sex cords are present with no thickening of the cortical layer. Scale bar = 50 μm. (**F**) Stage 19 male paramesonephric duct. Note the disorganization present in the epithelial ring and failure of the structure to detach from the kidney. Scale bar = 25 μm.

Subtle differences in diameter of the paramesonephric duct are present in different individuals, ranging from smaller than to approximately as large as the gonad in cross-section. As the duct develops, the frequency of histological sections lacking a lumen decreases.

At stage 19, the distinction between female and male gonads is clear in the majority of specimens

(6 of 8). The female gonad, in most cases, retains no indication of medullary sex cords (Fig. 1C). Germ cells are concentrated in the cortical region, which continues to have a darkly stained germinal epithelium 1–2 cells thick (Fig. 1C). Nearly the entire circumference (approximately one-half to three-fourths) of the paramesonephric duct of females is free of connection to the kidney (Fig. 1D). The nuclei in the epithelial cell lining are near the basal side.

In male gonads at stage 19, the sex cords are more distinct from the surrounding stroma (Fig. 1E). Germ cell concentrations are in the sex cords. The male paramesonephric duct may remain the same relative size as in the previous stage or decrease in size. The attachment to the kidney remains as it was in the prior two stages (i.e., with approximately half of the circumference attached) (Fig. 1F).

The gonad and paramesonephric duct of females at stage 20 remain essentially the same as described for the previous stage. The male gonad exhibits what appear to be vacant spaces developing around the sex cords in the medullary region. Male paramesonephric ducts are either completely absent or reduced in size with a collapsed lumen.

The gonads of both sexes at stage 21 (Fig. 2A) have developed a short mesenteric attachment to the kidney. Female gonads at this stage have a distinct cortico-medullary boundary, with the cortex at least two cells thick (Fig. 2A). The female paramesonephric duct has also developed a mesenteric attachment to the kidney at this stage. Some individuals appear to have two rings of columnar or cuboidal epithelial cells surrounding the increasingly large lumen in the duct. Opposite the mesenteric attachment to the kidney is a small conical protuberance (Fig. 2B), apparently identical to what Austin ('89) called a "mesenchymal ridge" in *Alligator mississipiensis*.

The male gonad at stage 21 retains a cortex that is 1–2 cells thick, which may contain a few germ cells in addition to those in the medullary sex cords. None of the male specimens retained a paramesonephric duct at stage 21.

At stage 22 in the female gonad, a distinct basement membrane is found at the cortico-medullary boundary. There are no germ cells remaining in the medullary region, which has begun to develop what appear to be vacant spaces as noted previously in male gonads. The seminiferous tubules (sex cords) of the male gonad become increasingly distinct from the surrounding connective tissue spaces of the medullary region by virtue of their darker staining cytoplasm as in Fig. 2E (at stage 23).

Spaces in the medullary region of stage 23 female gonads have increased (Fig. 2C). The male gonad is not significantly different from that of the previous stage (Fig. 2E). The "mesenchymal ridge" of the female paramesonephric duct has increased in size (i.e., it projects farther from the duct), and the cross-sectional profile of the duct is distinctly oval, as opposed to basically circular as it has been since its inception. Only one cell layer of columnar or cuboidal epithelium is present around the lumen, and the nuclei are darkly stained (Fig. 2D).

Gonads from embryos of stages 24 through 26 exhibit no additional distinctive changes. The female gonads appear much the same as those of stage 23. The seminiferous tubules of the male gonad continue to become increasingly distinct from the intervening connective tissue with advancing stages.

Additional changes in the paramesonephric ducts at stages 24–26 are negligible. We noted that at stage 26, the ring of epithelial cells surrounding the lumen of the paramesonephric duct in females appears to have an extremely well developed basement membrane at which the duct may cleave from the surrounding stroma when sectioned. The epithelial ring then appears as an insular body within the duct. Although this may be an artifact of preparation, the condition was present in all specimens examined.

Gonad length

Gonad lengths are compared for the two different incubation temperatures in Tables 1 and 2. Due to the small sample sizes for most stages, these data are presented to illustrate trends rather than draw statistical conclusions.

The indifferent gonads from stages 16–19 listed in Table 1 increased in length with each increment in stage. A noticeable difference in gonad length is evident between the two different incubation temperatures at each stage. In every case individuals incubated at 31°C have longer gonads than those incubated at 26°C.

For stages 19 through 26, the average gonad length increased with each stepwise increase in stage (Table 2). In all possible pairwise comparisons at a particular temperature and stage, female gonads are longer than those of males. Also, a trend is noticeable within stages for both testes and ovaries to have greater average length at the

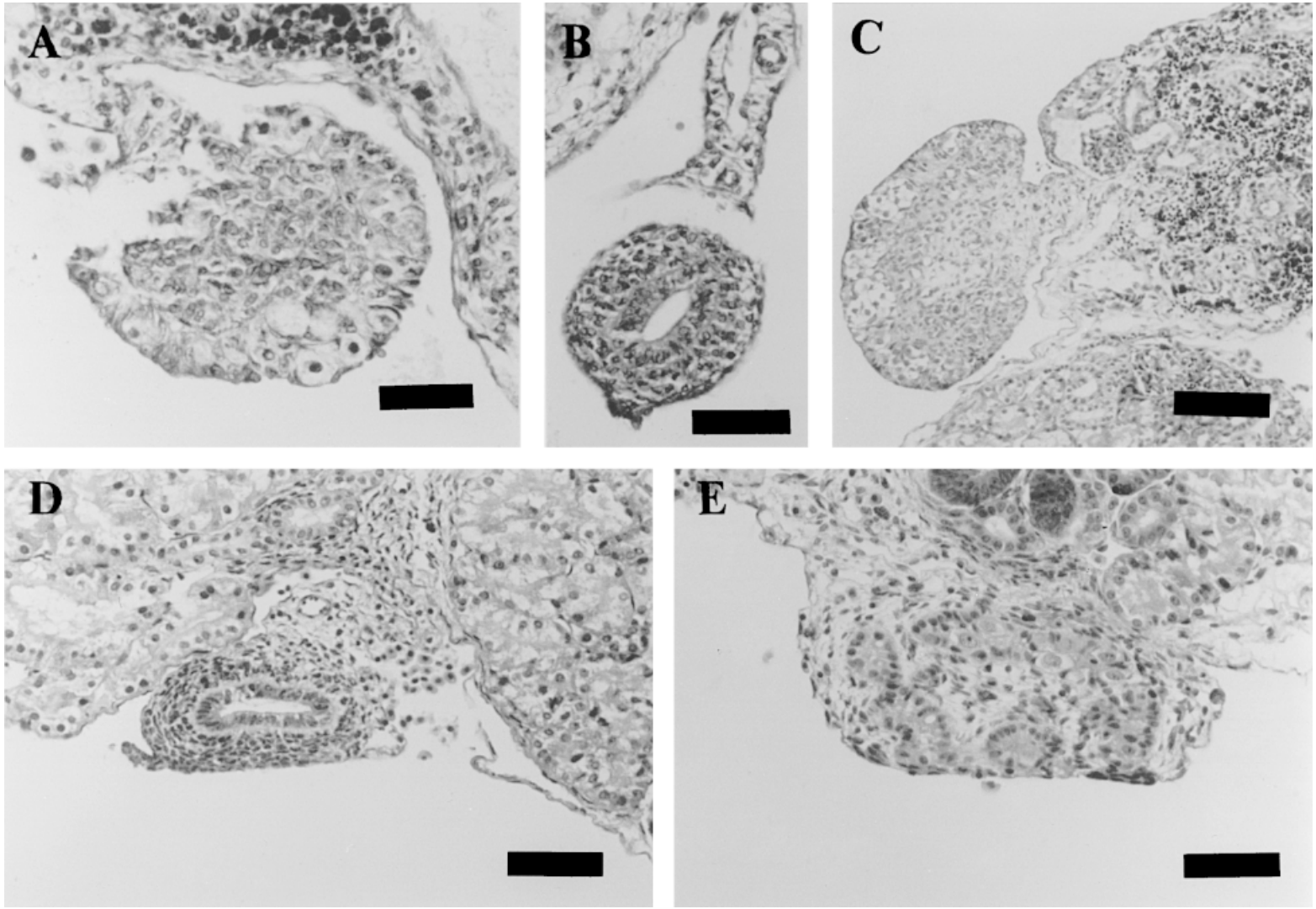

Fig. 2. (**A**) Stage 21 female gonad. Note the prominent mesenteric attachment to the kidney. The distinction between the cortex and medulla is noticeable. Scale bar = 50 μm. (**B**) Stage 21 female paramesonephric duct. Note the prominent mesenteric attachment to the kidney and the appearance of a small mesenchymal ridge. Scale bar = 50 μm. (**C**) Stage 23 female gonad. Scale bar = 100 μm. (**D**) Stage 23 female paramesonephric duct. Note the increase in length of the mesenchymal ridge. Scale bar = 50 μm. (**E**) Stage 23 male gonad. Note that it is lying next to the kidney, not attached along its entire length. Scale bar = 50 μm.

TABLE 1. Average length of indifferent gonads in A. spinifera *embryos at 26°C and 31°C*[1]

Stage	26°C	31°C
16	1.53 (3) ± 0.41	1.66 (4) ± 0.28
17	1.67 (1)	1.72 (5) ± 0.40
18	1.85 (3) ± 0.02	1.90 (4) ± 0.22
19[2]	2.26 (4) ± 0.53	2.52 (4) ± 0.51

[1]Data are average lengths in mm (sample size) ± 1 SD.
[2]Only two specimens from stage 19 were sexually indifferent, but all speciments are included in the table to facilitate comparison.

warmer incubation temperature, except at stage 21 (Table 2). Due to the small sample sizes in the cells of the sex by temperature by stage data matrix, statistical analyses of the aforementioned trends were not attempted.

DISCUSSION

Gonadal differentiation

The bisexual characteristics of gonads prior to sexual differentiation are typical for vertebrates and have been noted in many groups of reptiles (Fox, '77; Raynaud and Pieau, '85). The indifferent characteristics of simultaneous possession of medullary sex cords and a thickened cortical layer of the gonad, as well as the random localization of germ cells has been noted in the turtles *Sternotherus odoratus*, *Emys orbicularis*, *Testudo graeca* and *Trachemys scripta* (Risley, '33; Raynaud and Pieau, '85; Wibbels et al., '91a).

Although nuances of the path of sexual differentiation were evident in specimens of *Apalone spinifera* at stage 18, gonads were not assigned to a particular sex until stage 19 (Fig. 1C and E). Ewert ('85) noted that sex specific changes were evident in stage 17 embryos of *Emys orbicularis* incubated at different temperatures. However, in that species as well as *Testudo graeca* and *Chrysemys picta*, a definite sex was not assigned until stage 19+ or 20. In the emydid *Trachemys scripta*, Wibbels et al. ('91a) noted that sex specific changes began to occur between stages 18–20, but they did not indicate that they were definitive until stage 23. Risley ('33) used measurements to stage the embryos he examined, which complicates stagewise comparison with other taxa, but it was noted that embryos between 10.0 and 14.0 mm in carapace length could be characterized as inherently male, female, or bisexual according to development of the sex cords and epithelial cortex of the gonad. Presumably irreversible differentiation of gonads into ovaries or testes occurs in *Apalone spinifera* embryos at stage 19 of development (Fig. 1C and E).

Ovaries

Risley ('33) first mentioned an ovary of *Sternotherus odoratus* in 14.0 mm carapace length embryos. He described regression of the sex cords as the main characteristic of these embryos, which presumably coincides with the observed rapid disappearance of sex cords in *Apalone spinifera* female gonads. However, Risley ('33) reported that germ cells were not present in the cortex, but continued to linger in the medullary area of the gonad in contrast to the findings of this study. Wibbels et al. ('91a) described the gradual regression of medullary cords in *Trachemys scripta* between stages 18–20, but also mentioned a distinct boundary between the cortex and medulla, which was not seen uniformly in all *Apalone spinifera* until stage 22 (Fig. 2C). However, this latter characteristic was variable and its presence may have been masked by differences in staining techniques.

In *Sternotherus odoratus* embryos that had reached 16.6 mm in carapace length, Risley ('33) discussed germ cell activity in the cortex and the rapid reduction in the relative extent of the medullary layer. Although no explanation accompanied

TABLE 2. Average length of gonads in sexually diagnosable A. spinifera *embryos at 26°C and 31°C*[1]

	26°		31°	
Stage	♂	♀	♂	♀
19[2]	1.98 (1)	2.62 (2) ± 0.54	2.13 (2) ± 0.28	2.65 (1)
20	1.83 (3) ± 0.37	2.36 (1)	1.94 (2) ± 0.08	2.94 (1)
21	2.77 (5) ± 0.43	3.26 (3) ± 0.52	2.42 (7) ± 0.42	3.03 (8) ± 0.86
22	2.53 (1)	—	2.87 (2) ± 0.23	3.10 (3) ± 0.79
23	—	—	2.14 (3) ± 0.63	3.49 (2) ± 0.72
24	2.80 (1)	—	2.85 (2) ± 0.31	3.49 (2) ± 0.31
25	2.97 (1)	3.50 (2) ± 1.10	3.27 (1)	3.72 (3) ± 1.35
26	—	3.53 (2) ± 0.94	2.20 (4) ± 0.58	5.02 (2) ± 0.33

[1]Data are average lengths in mm (sample size) ± 1 SD.
[2]The two sexually indifferent specimens from stage 19 included in Table 1 are omitted here.

it, a photograph illustrating the mesenteric attachment of the gonad to the kidney was shown in an embryo with a carapace length of 22.0 mm. Since no mention of its genesis was mentioned, no comparison can be made to *Apalone spinifera*. However, Wibbels et al. ('91a) mentioned the structure at stage 21, which is the same stage that it appears in the softshell turtle (Fig. 2A). The finding that the cortex was three cells thick at this stage in *Trachemys scripta* agrees with the chronology in *Apalone spinifera* (Fig. 2A).

Beyond this, it was mentioned that the medulla regressed and the cortex advanced with an abundance of germ cell activity from stages 21–26 in *Trachemys scripta*. This also is in agreement with the ovarian development of *Apalone spinifera* since the variation in apparent decrease in relative medullary area was too great among individuals to attribute to a particular stage. Within ten days before hatching, Risley ('33) noted that oocytes within the ovary began to grow to a marked degree in *Sternotherus odoratus*. We did not observe oocyte proliferation in embryonic specimens of *Apalone spinifera*, nor was it noted in *Trachemys scripta* (Wibbels et al., '91a).

Testes

In describing development of the testis, Risley ('33) noted that variation among individuals was too great to attribute sex cord proliferation and cortical reduction to a particular embryonic stage. His only stage specific comment referred to the nearly complete regression of the cortex in the testis by the time of hatching. Wibbels et al. ('91a) noted membranes around the sex cords at stages 18–19 in *T. scripta*. Such membranes were variable in appearance in *Apalone spinifera*, but were evident in some specimens as early as stage 19. The sex cords contained germ cells between stages 18–20 in *T. scripta,* which coincides with events in the softshell turtle testis.

As mentioned for the ovary at stage 21, the testis also developed a mesenteric attachment to the kidney in *T. scripta* at that stage (Wibbels et al., '91a). This is synchronous with the development of that structure observed in *A. spinifera*. Between stages 21–26, the seminiferous tubules enlarged within the medulla while the cortical epithelium remains approximately the same thickness in the emydid just as in the softshell turtle, but a definitive one-cell thick germinal epithelium during this period was not uniformly present in *A. spinifera*. Many individuals retained a two cell thick epithelial covering.

Paramesonephric ducts (Müllerian ducts)

In reptiles generally, the paramesonephric ducts develop in a craniocaudal direction (Raynaud and Pieau, '85). Raynaud and Pieau ('85) reported that the ostium tubae of the forming paramesonephric ducts are present at stage 13–14 of the tortoise *Testudo graeca*. Ewert ('85) noted that the Müllerian duct advances posteriorly from its attachment to the kidney, reaching halfway down the length of the kidney at stage 17 and the cloaca by stage 20 in the same species. Wibbels et al. ('99) outlined the development of the Müllerian ducts in the red-eared slider (*T. scripta*). In that species, the ducts developed in a craniocaudal direction and had reached the level of the gonad by stages 18–19. By comparison, we found that in *A. spinifera*, the Müllerian duct had reached the level of the gonad by stage 16 and had a well-developed lumen with an epithelial ring in nearly all sections by stages 17 and 18. All our observations of Müllerian ducts were made in cross-sections that included the gonad, thus we did not record the direction of duct formation.

Regression of the Müllerian ducts in male reptiles follows differentiation of the testis (Raynaud and Pieau, '85; Austin, '89; Wibbels et al., '99). In both mammals and chickens, Müllerian inhibiting substance (MIS) produced by the newly differentiated testis causes this regression (see review in Lee and Donahoe, '93). Although it remains to be demonstrated, it is hypothesized that MIS is involved in reptilian Müllerian duct regression as well (Wibbels et al., '98, '99).

The complete absence of a paramesonephric duct after stage 20 in males of *A. spinifera* has not been reported for a turtle species. Fox ('77) noted that depleted Müllerian ducts were present in young males of *Emydoidea blandingii* and *Mauremys caspica*. Pieau et al. ('98) found the ducts degenerated between stages 23–25 in the emydid *Emys orbicularis*. Wibbels et al. ('99) found distinct regression of the ducts at stages 22–23 in male *T. scripta*; by stage 26 they were absent. In the green turtle, *Chelonia mydas*, a degenerate paramesonephric duct was still present in males at stage 26 (Miller and Limpus, '81). Risley ('33) illustrated a degenerate oviduct (Müllerian duct) at stage 26 of *Sternotherus odoratus* (Kinosternidae). Although a remnant paramesonephric duct with a small lumen is illustrated for a stage 26 male specimen of *Carettochelys insulpta* (Carettochelyidae), Webb et al. ('86) noted that the duct has often completely degenerated in many speci-

mens by the time of hatching. Since the Carettochelyidae is the sister group to the Trionychidae (Gaffney and Meylan, '88; Shaffer et al., '97), it is feasible that paramesonephric duct degeneration occurs more completely in this clade of turtles than in more distantly related taxa.

In *A. spinifera*, the paramesonephric ducts developed to the same extent in all embryos between stages 16 through 18, but some degeneration was noted in males at stage 19. Stage 20 males all had regressing ducts, and they could not be found in any stage 21 males. Gonadal differentiation is primarily occurring at stage 19, thus Müllerian duct regression follows very closely on the appearance of differentiating testes and is complete at a relatively early point prior to hatching.

Gonadal length

Risley ('33) demonstrated an increasing sexual divergence in embryonic gonad length as embryonic size increased in *S. odoratus*. Wibbels et al. ('91b) measured gonads of hatchling *Graptemys caglei* and noted significant differences between ovary and testis length. In addition, sample sizes were large enough to confirm statistically significant differences in ovary length between individuals incubated at 29.0–29.5°C and those incubated at 30.5°C. Gonad lengths were measured at stages 22 and 26 in *T. scripta* with statistically different average lengths for testes and ovaries (Wibbels et al., '91a). Once again, a statistically significant difference in ovary lengths was found between embryos incubated at two temperatures (29°C and 31°C) (Wibbels et al., '91a). The very same trends are present in our *Apalone* data, i.e., ovaries are longer than testes at each stage for each of the two temperatures. Although sample sizes are limited, ovaries are longer at the warmer temperature (31° vs. 26°C) for all stages except one (Table 2). Sample sizes were not sufficient for robust testing of significance in the gonad lengths of males and females at equivalent stages, but the trend in the data is consistent and coincides with the established trend of ovaries being longer than testes (Risley, '33; Wibbels et al., '91a,b).

EVOLUTIONARY IMPLICATIONS

Knowing the chronology of sexual differentiation in a turtle species with GSD allows for comparison with TSD species and may help elucidate the evolutionary significance of sex determination mode. Although sexual differentiation seems to take place at the same time in embryological development in many taxa of turtles, the fact that with GSD sex is irreversible at this point is a significant departure from the situation in TSD species in which the differentiating gonad is labile with respect to temperature. Embryos of *Chelydra serpentina* can be influenced by temperature until stage 19 (Yntema, '79), *Trachemys scripta* to stage 20 (Wibbels et al., '91a), and *Graptemys*, *Chrysemys*, *Emys* and *Caretta* to stage 22 (Bull and Vogt, '81; Pieau and Dorizzi, '81; Yntema and Mrosovsky, '82). In nearly all these turtle taxa, temperature can alter the path of gonadal differentiation after the time at which *A. spinifera* embryonic gonads have definitively entered a developmental path toward an ovary or testis (stage 19). The absolute nature of the developmental path taken in *A. spinifera* may be reflected in the rapid degeneration of the male Müllerian ducts following testicular differentiation, since duct regression is hypothetically due to a putative testicular hormone (MIS).

There is no fundamental difference in the sequence or timing of events in the gonadogenesis of *A. spinifera* as compared to TSD species of turtles, and therefore it cannot be argued that this study provides strong support of the corollary hypothesis of Charnov and Bull ('77). The hypothetical expectation is that GSD species will differentiate sexually earlier than TSD species because they have no adaptive advantage in postponing the process. In fact, it may be advantageous to differentiate earlier in order to reach sexual maturity earlier and thereby enhance reproductive fitness (Charnov and Bull, '77). This prediction of the Charnov-Bull model may not be reflected in embryonic development and therefore not testable at the level of primary gonadal differentiation in turtles for at least the following two reasons: (1) gonadal differentiation may be strongly canalized and it occurs in all taxa early, at close to the same stage of development, and is thus maintained by stabilizing selection; or (2) because embryonic development is so short compared to chelonian life spans, heterochrony in gonadal differentiation may have no effect on the ultimate time of sexual maturation, and therefore no impact on lifetime reproductive fitness.

Sexual dimorphism in size and color pattern is pronounced in adults of *A. spinifera* (Webb, '62). Secondary sexual characteristics in coloration have been reported from hatchlings and young juveniles of *A. s. spinifera* (Graham, '91; Graham and Cobb, '98), and *A. s. asper* (Webb, '62), and is known in other subspecies as well (Ewert and Vogt, personal communication; Carr, personal observation). The

significance of this early onset of secondary sexual characteristics is unknown, but since this dichromatism has been reported in hatchlings (stage 26), it must necessarily be the consequence of embryonic events. Secondary sexual characteristics in vertebrates are usually associated with the onset of sexual maturity and the production of sex steroids (van Tienhoven, '83). Additional study of *A. spinifera* will be required in order to relate the embryonic events of primary sexual differentiation, precocious onset of secondary sexual characteristics, and the ultimate attainment of reproductive maturity.

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LITERATURE CITED

Austin HB. 1989. Müllerian-duct regression in the American Alligator (*Alligator mississipiensis*): its morphology and testicular induction. J Exp Zool 251:329–338.

AVMA Panel on Euthanasia. 1993. Report of the AVMA Panel on Euthanasia. J Am Vet Med Assoc 202:229–249.

Bickham JB, Bull JJ, Legler JM. 1983. Karyotypes and evolutionary relationships of trionychoid turtles. Cytologia 48:177–183.

Bull JJ, Vogt RC. 1979. Temperature-dependent sex determination in turtles. Science 206:1186–1188.

Bull JJ, Vogt RC. 1981. Temperature-sensitive periods of sex determination in emydid turtles. J Exp Zool 218:435–440.

Bull JJ, Legler JM, Vogt RC. 1985. Nontemperature dependent sex determination in two suborders of turtles. Copeia 1985:784–786.

Bull JJ, Gutzke WHN, Crews D. 1988. Sex reversal by estradiol in three reptilian orders. Gen Comp Endocrin 70: 425–428.

Charnov EL, Bull JJ. 1977. When is sex environmentally determined? Nature 266:828–830.

Ewert MA. 1985. Embryology of turtles. In: Gans C, Billett F, Maderson PFA, editors. Biology of the reptilia, Vol. 14. New York: John Wiley & Sons, Inc., p 75–268.

Ewert MA, Jackson DR, Nelson CE. 1994. Patterns of temperature-dependent sex determination in turtles. J Exp Zool 270:3–15.

Ewert MA, Nelson CE. 1991. Sex determination in turtles: diverse patterns and some possible adaptive values. Copeia 1991:50–69.

Fox H. 1977. The urinogenital system of reptiles. In: Gans C, editor. Biology of the reptilia, Vol. 6. New York: Academic Press Inc., p 1–122.

Gaffney ES, Meylan PA. 1988. A phylogeny of turtles. In: Benton MJ, editor. The phylogeny and classification of tetrapods. Oxford, England: Clarendon Press, p 157–219.

Georges A. 1988. Sex determination is independent of incubation temperature in another chelid turtle, *Chelodina longicollis*. Copeia 1988:248–254.

Graham TE. 1991. Life history notes: *Apalone spinifera spinifera* (Eastern Spiny Softshell). Pattern dimorphism. Herpetol Rev 22:97.

Graham TE, Cobb CB. 1998. Sexual dimorphism of neonate Eastern Spiny Softshells, *Apalone spinifera spinifera*. Chel Conserv Biol 3:111–112.

Janzen FJ, Paukstis GL. 1991. Environmental sex determination in reptiles: ecology, evolution, and experimental design. Q Rev Biol 66:149–179.

Lance VA. 1997. Sex determination in reptiles: an update. Am Zool 37:504–513.

Lance VA, Bogart MH, editors. 1998. Proceedings from the first international symposium on vertebrate sex determination. J Exp Zool 281:357–528.

Lang JW, Andrews H, Whitaker R. 1989. Sex determination and sex ratios in *Crocodylus palustris*. Am Zool 29:935–952.

Lee MM, Donahoe PK. 1993. Müllerian inhibiting substance: a gonadal hormone with multiple functions. Endo Rev 14:152–164.

Marquez E, Denardo D, Hayes TB. 1999. Sex determination and sex differentiation in reptiles. Am Zool 39:22A.

McBee K, Bickham JW, Rhodin AGJ, Mittermeier RA. 1985. Karyotypic variation in the genus *Platemys* (Testudines: Pleurodira). Copeia 1985:445–449.

Miller JD, Limpus CJ. 1981. Incubation period and sexual differentiation in the green turtle *Chelonia mydas* L. In: Banks CB, Martin A, editors. Proceedings of the Melbourne herpetological symposium. Melbourne: The Royal Melbourne Zoological Gardens, p 66–73.

Pieau C. 1996. Temperature variation and sex determination in reptiles. BioEssays 18:19–26.

Pieau C, 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.

Pieau C, Dorizzi M, Richard-Mercer N, Desvages G. 1998. Sexual differentiation of gonads as a function of temperature in the turtle *Emys orbicularis*: endocrine function, intersexuality and growth. J Exp Zool 281:400–408.

Presnell JK, Schreibman MP. 1997. Humason's animal tissue techniques, 5th ed. Baltimore, MD: The Johns Hopkins University Press.

Raynaud A, Pieau C. 1985. Embryonic development of the genital system. In: Gans C, Billett F, editors. Biology of the reptilia, Vol. 15. New York: John Wiley & Sons, Inc., p 149–300.

Risley PL. 1933. Contributions on the development of the reproductive system in the musk turtle, *Sternotherus odoratus* (Latreille). II. Gonadogenesis and sex differentiation. Z Zellforsch 18:493–541.

Shaffer, HB, Meylan P, McKnight ML. 1997. Tests of turtle phylogeny: molecular, morphological, and paleontological approaches. Syst Biol 46:235–268.

Thompson MB. 1988. Influence of incubation temperature and water potential on sex determination in *Emydura macquarii* (Testudines: Pleurodira). Herpetologica 44:86–90.

van Tienhoven A. 1983. Reproductive physiology of vertebrates, 2nd ed. Ithaca, NY: Cornell University Press.

Vogt RC, Bull JJ. 1982. Genetic sex determination in the spiny softshell turtle *Trionyx spiniferus* (Testudines: Trionychidae) (?). Copeia 1982:699–700.

Webb GJW, Choquenot D, Whitehead PJ. 1986. Nests, eggs and embryonic development of *Carettochelys insculpta* (Chelonia:Carettochelidae) from northern Australia. J Zool B 1:521–550.

Webb RG. 1962. North American recent soft-shelled turtles (Family Trionychidae). University of Kansas Publications, Mus Nat Hist 13:429–611.

Wibbels T, Bull JJ, Crews D. 1991a. Chronology and morphology of temperature-dependent sex determination. J Exp Zool 260:371–381.

Wibbels T, Killebrew FC, Crews D. 1991b. Sex determination in Cagle's map turtle: implications for evolution, development, and conservation. Can J Zool 69:2693–2696.

Wibbels T, Bull JJ, Crews D. 1994. Temperature-dependent sex determination: a mechanistic approach. J Exp Zool 270:71–78.

Wibbels T, Cowan J, LeBoeuf R. 1998. Temperature-dependent sex determination in the red-eared slider turtle, *Trachemys scripta*. J Exp Zool 281:409–416.

Wibbels T, Wilson C, Crews D. 1999. Müllerian duct development and regression in a turtle with temperature-dependent sex determination. J Herpetol 33:149–152.

Yntema CL. 1968. A series of stages in the embryonic development of *Chelydra serpentina*. J Morphol 125:219–251.

Yntema CL. 1979. Temperature levels and periods of sex determination during incubation of eggs of *Chelydra serpentina*. J Morphol 159:17–28.

Yntema CL. 1981. Characteristics of gonads and oviducts in hatchlings and young of *Chelydra serpentina* resulting from three incubation temperatures. J Morphol 167:297–304.

Yntema CL, Mrosovsky N. 1982. Critical periods and pivotal temperatures for sexual differentiation in loggerhead sea turtles. Can J Zool 60:1012–1016.