Zoological Studies **59:**20 (2020)
doi:10.6620/ZS.2020.59-20

Zoological Studies

Open Access

Chronology of Gonadal Development in the Malayan Snail-eating Turtle *Malayemys macrocephala*

Rangsima Pewphong[1], Jirarach Kitana[1,2], and Noppadon Kitana[1,2,*]

[1]*Department of Biology, Faculty of Science, Chulalongkorn University, Bangkok 10330, Thailand.*
**Correspondence: E-mail: noppadon.k@chula.ac.th (NK)*
E-mail: airr_rsm@hotmail.com (Pewphong); jirarach.s@chula.ac.th (JK)
[2]*BioSentinel Research Group (Special Task Force for Activating Research), Department of Biology, Faculty of Science, Chulalongkorn University, Bangkok 10330, Thailand*

Received 6 July 2019 / Accepted 8 May 2020 / Published 17 June 2020
Communicated by Benny K.K. Chan

The snail-eating turtle, *Malayemys macrocephala*, is a common freshwater turtle that can be used as an animal model for developmental biology. However, a thorough investigation of its development is needed before this species can be used as a model. Thus, this study aimed to examine the gonadal development of *M. macrocephala*. Turtle eggs were collected from rice fields in Phra Nakhon Si Ayutthaya Province, Thailand, and transported to the laboratory. Eggs were incubated in microprocessor-controlled incubators and randomly dissected on a weekly basis to reveal the developing embryos, then their developmental stage was identified according to Yntema (1968). Primordial germ cells and gonad structure were processed through the paraffin method. Moreover, the dynamics of germ cell proliferation and apoptosis were examined by immunohistochemical detection of proliferating cell nuclear antigen (PCNA) and the terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL), respectively. Examination of the gonad revealed four main stages of gonadal development: (i) germ cell migration, (ii) genital ridge appearance, (iii) testicular formation, and (iv) ovarian formation. In the male turtle (incubated at 26°C), gonad developed into the testis with medullary sex cords starting at Yntema stage 17. In the female turtle (incubated at 32°C), these sex cords then degenerated, followed by cortical development into an ovarian structure starting at Yntema stage 19. Subsequently, testicular and ovarian development occurred independently, and distinct sex organs were apparent at Yntema stage 25. In addition, the presumptive testis showed germ cell proliferation in the medulla at Yntema stages 17, 19, and 25 and germ cell apoptosis in the cortex at Yntema stages 19 and 25. The presumptive ovary showed germ cell proliferation in the cortex at Yntema stages 19 and 25, and germ cell apoptosis in the medulla at Yntema stages 19 and 25.

Key words: Apoptosis, Embryos, Freshwater turtle, Microscopic examination, Proliferation.

BACKGROUND

Sex determination in reptiles is mediated via temperature-dependent sex determination (TSD), which provides an ideal model system to test predictions concerning the biological importance of global temperature change (Girondot and Kaska 2014). The first study on gonadal development in freshwater turtles showed qualitative differences in response to different incubation temperatures (Ewert 1985). In many turtles, gonad sex differentiation is controlled by the incubation temperature of the egg during a critical period of embryonic development (Pieau and Dorizzi 1981). The time during embryonic development when

Citation: Pewphong R, Kitana J, Kitana N. 2020. Chronology of gonadal development in the Malayan snail-eating turtle *Malayemys macrocephala*. Zool Stud **59:**16. doi:10.6620/ZS.2020.59-20.

the temperature influences the sexual phenotype of the gonad is referred to as the temperature sensitive period (TSP) (Girondot et al. 2018).

The somatic development of turtle embryos is generally established based on morphological changes in 26 stages starting from gastrulation to hatching (Yntema 1968). Gonadal development is one of the important landmarks in organogenesis and can be divided into four phases. The first is the germ cell migration phase, when the germ cells in the posterior germinal crescent at the end of gastrulation migrate through the dorsal mesentery to reach the gonad anlagen (Pieau et al. 1999). A prior study on the mechanism by which the primordial germ cells (PGCs) migrate was evaluated by light microscopy in the turtle (Ewert 1985), in which the PGCs were located in the posterior part of the extra embryonic area and then migrated by ameboid movement, similar to germ cell migration in other vertebrates (Ewert 1985). The second phase is the bipotential gonad phase, when the bipotential genital ridge forms and both sexes develop primitive medullary sex cords (Yntema stages 14 and 15). The third phase is the sex determination phase, when the gonad is differentiated but the sexual trend of the gonadal primordium is still flexible (Yntema stages 16–19). The fourth is the sex differentiation phase, when the final gonadal morphology develops and the sex is fully determined (testicular or ovarian structures form; Yntema stages 20–26) (Yao et al. 2004).

In the slider turtle (*Trachemys scripta*), the gonad develops from the genital ridge and can be divided into two zones: an inner medulla and outer cortical layers. Primitive sex cords are found in the medullary region of the bipotential gonad, regardless of the environmental temperature. In the developing ovary, these primitive sex cords degenerate during stages 18–20, while in the testis they develop into the seminiferous tubules starting at stage 18 and become very evident by stage 20 (Ramsey and Crews 2009). Interestingly, the cords develop into the testis while the sex is still labile, making this process reversible until about stages 20–21 (Ramsey and Crews 2009). For gonad development, germ cells are initially located in the outer germinal epithelium of the gonad. In the ovary, the germ cells remain in the cortical region, where they proliferate along with differentiating granulosa cells to form follicles. In the developing testis, the germ cells migrate into the medullary region beginning at stage 18, where they are enveloped by the developing seminiferous tubules (Wibbels et al. 1991; Yao et al. 2004).

Although 16 species (two families) of freshwater turtles can be found in Thailand (Pipatsawasdikul et al. 2010; Chan-ard et al. 2015; Ihlow et al. 2016), their susceptibility to temperature change is unknown due to a lack of information on the chronology of their gonadal development pattern. The snail-eating turtle, *Malayemys macrocephala*, is a native freshwater turtle commonly found in rice fields in central Thailand. Previous histological analysis of this species suggested that TSD occurs in males at 26°C and females at 32°C (Pewphong et al. 2016). This study aimed to examine the chronology of gonadal development in *M. macrocephala* using histological analysis during the germ cell migration, bipotential, sex determination, and sex differentiation phases. Moreover, germ cell dynamics were followed by immunohistochemical staining for proliferating cell nuclear antigen (PCNA) and terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) to detect cell proliferation and apoptosis, respectively, in *M. macrocephala*.

MATERIALS AND METHODS

Turtle egg collection

All egg collection and handing procedures were approved by the Chulalongkorn University Animal Care and Use Committee (Protocol Review Number 1723005), and eggs were collected in accordance with the Wild Animals Reservation and Protection Act B.E. 2546 of Thailand and approved by the Department of Fisheries, Ministry of Agriculture and Cooperatives (Permit Number 17/2559). Turtle eggs were collected from an agricultural area of Bang Ban District, Phra Nakhon Si Ayutthaya Province, central Thailand (UTM Zone 47P 0650143-0663244 and 1586924-1599393), which have large areas of *M. macrocephala* habitat and nesting sites (Keithmaleesatti 2008). Turtle nests were found by visual searching from late November 2016 to April 2017, and a total of 570 eggs from 95 clutches were located. Only freshly laid eggs without white spot development were collected. These eggs were labeled individually before being transported to a laboratory at Chulalongkorn University, Bangkok, Thailand.

Turtle egg incubation

Eggs from each clutch were randomly assigned to covered boxes containing moistened vermiculite, which were then divided into two groups to be incubated at either 26°C, the male producing temperature (MPT), or 32°C, the female-producing temperature (FPT). The egg trays were rotated twice a week within the incubator to avoid small temperature-gradient effects. Three eggs per group of the same age, laid on the same day, were randomly selected and dissected on a weekly basis. After dissection, the developmental stage of the turtle

embryo was determined according to their external morphology. The series of normal developmental stages of the snail-eating turtle *M. macrocephala* used in this study is comparable to the widely used developmental stages of *Chelydra serpentina* (Yntema 1968) and *Pelodiscus sinensis* (Tokita and Kuratani 2001), with a minute difference in that *M. macrocephala* lacks the early stages (Yntema stages 1–5) (Pewphong 2012).

Histology of gonadal development

To study the germ cell migration, embryos from MPT and FPT rearings were collected from Yntema stages 10 to 14 and their gonad structure was histologically examined from Yntema stages 14 to 25. Embryos were immersed in neutral buffer formalin fixative (3–6 embryos per stage per temperature) for 24 h and then preserved in 70% (v/v) ethanol. Fixed embryos (Yntema stages 10–19) and dissected gonads (Yntema stages 20–25) were embedded in paraffin, sectioned at 5 μm thickness, and periodic acid Schiff stained before being observed using light microscopy. Gonadal development and sex differentiation in *M. macrocephala* were evaluated with reference to previous reports on the snapping turtle *Trachemys scripta* and the Magdalena river turtle, *Podocnemis lewyana* (Wibbels et al. 1991; Sánchez et al. 2014).

Proliferating cell detection (PCNA immunohistochemical assay)

Malayemys macrocephala embryos (stages 15, 17, 19) and gonads (stage 25) were processed by the paraffin method and sectioned in a transverse plane at 5 μm thickness and immunohistochemically stained for proliferating cell nuclear antigen (PCNA), a marker for cell division (Wolf and Dittrich 1992). The PCNA is a ring-like protein that provides the DNA polymerase processivity for DNA replication (Kelman 1997). The sections were deparaffinized, hydrated, and incubated in two changes of 3% (v/v) H_2O_2 for 10 min. After rinsing in distilled water, slides were pretreated with 1% (w/v) zinc sulfate in a 800 W microwave oven at 100% power setting for 15 min to retrieve the antigenic determinants. Sections were washed in distilled water, followed by 0.05% (v/v) Tween-20 in phosphate buffered saline (PBS-T). Nonspecific binding was blocked by adding 3% (v/v) normal horse serum to each slide and incubating at room temperature for 1 h. Slides were washed between each incubation step in PBS-T. Sections were incubated with mouse monoclonal antibody against PCNA (Bioscience International) overnight at 4°C, then washed and incubated with biotinylated horse anti-mouse IgG (Millipore Corporation) for 1 h. After washing, they were incubated for 10 min with 0.03% (w/v) 3,3'-diamionobenzidine-4 HCl (DAB; Sigma-Aldrich), 3% (v/v) H_2O_2, and 0.2% (w/v) ammonium nickel sulfate in PBS, then rinsed with distilled water, dehydrated, cleared, and mounted with Permount™.

Apoptosis detection (TUNEL assay)

Malayemys macrocephala embryos (stages 15, 17, and 19) and gonads (stage 25) were processed by the paraffin method and sectioned in a transverse plane to 5 μm thick. Apoptotic cells in the gonads were then detected by the TUNEL assay (Gavrieli et al. 1992) using a DNA fragmentation kit (Merck Millipore, catalog number S7107). TUNEL is based on the ability of TdT to label blunt ends of double-stranded DNA breaks independent of a template (Kyrylkova et al. 2012). Each paraffin section of the gonad was deparaffinized, hydrated, and incubated in proteinase K for 15 min at room temperature to permeabilize the specimen, followed by 3% (v/v) H_2O_2 in methanol for 15 min to block endogenous peroxidase activity. After incubating with equilibration buffer for 10 s, fragmented DNA was labeled by incubating with the TdT enzyme in the TdT reaction mixture at 37°C for 1 h. The reaction was terminated by submerging the sections in 10 mM EDTA solution. The sections were then incubated with blocking buffer for 10 min, and labeled DNA was detected by incubating the sections with peroxidase streptavidin conjugate for 30 min, then washed and developed in 0.03% (w/v) DAB and 3% (v/v) H_2O_2 in PBS. Slides were then rinsed in distilled water, counterstained with 0.5% (w/v) methyl green, dehydrated, cleared, and mounted with Permount™.

RESULTS

Chronology of gonadal development

The chronology of gonadal development is summarized in table 1. The embryonic developmental rate, as assessed by morphological stages, was faster when the embryo was incubated at the FPT (32°C) than at the MPT (26°C), as expected. Microscopic examination of the gonad revealed four main stages of gonadal development: germ cell migration (Fig. 1), genital ridge appearance, testicular formation, and ovarian formation (Fig. 2).

Early gonad development (Yntema stages 10 to 14) was examined to follow the germ cell migration, and the histological study of PGCs in *M. macrocephala* was based on a previous report on the loggerhead turtle (*Caretta caretta*; Fujimoto et al. 1979). The PGCs

appeared at stage 10 (day 14 and 24 at the FPT and MPT, respectively) outside the genital ridge or a ridge of embryonic mesoblast developing from the mesonephros and giving rise to the gonad on either side of the body (Fig. 1a). They were observed migrating from the yolk sac into the connective tissue of the hindgut at stage 11 (day 17 and 28 at the FPT and MPT, respectively; Fig. 1b). At stage 12 (day 18 and 32 at the FPT and MPT, respectively), the PGCs migrated from the hindgut to the mesonephric duct (Fig. 1c), and then higher in the mesonephric duct at stage 13 (day 19 and 36 at the FPT and MPT, respectively) (Fig. 1d). Similarly, the transverse section of *M. macrocephala* embryos at stage 14 (day 22 and 40 at the FPT and MPT, respectively)

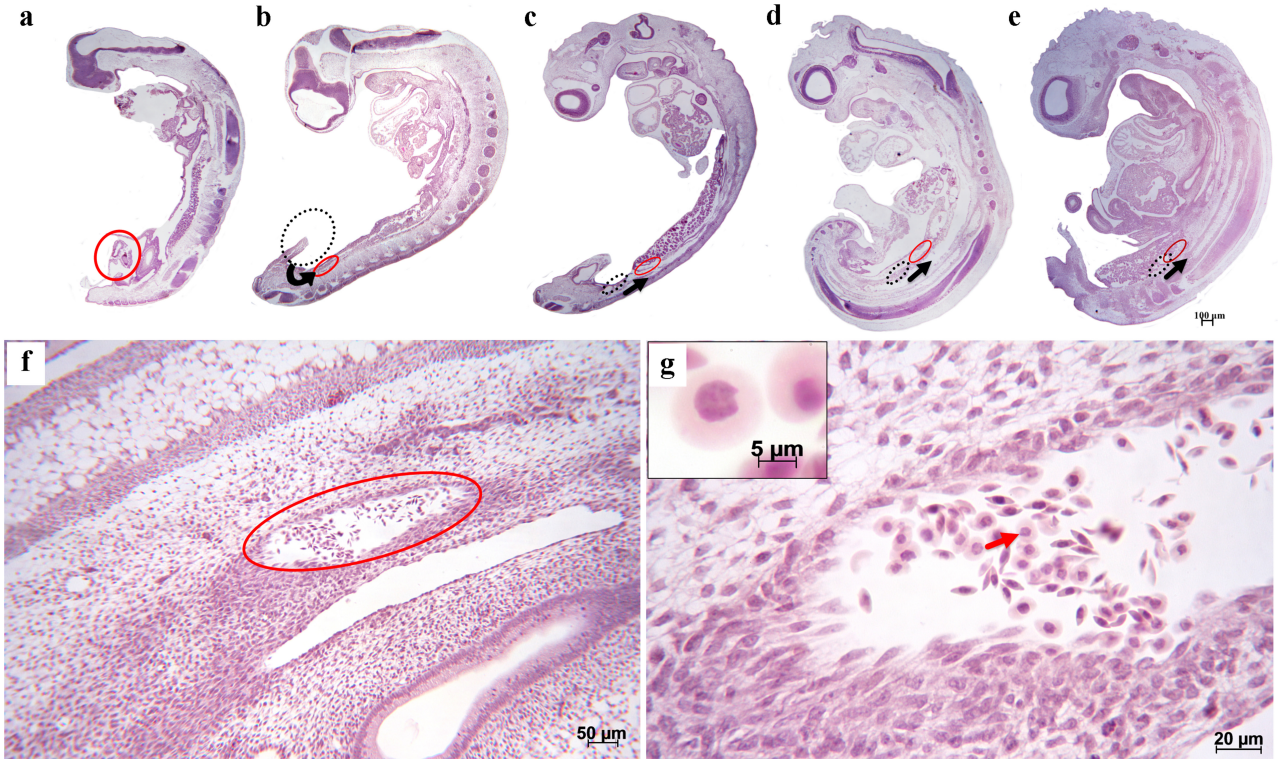

Fig. 1. Location of the PGCs in *M. macrocephala* embryos. The red oval line indicates the location of the PGCs at the current stage, while the black oval dashed line indicates their previous location in embryos at stage (a) 10, (b) 11, (c) 12, (d) 13, and (e) 14, plus the (f) area and (g) character of the PGCs, which can be identified by its large size, round nuclei, and pale cytoplasm (red arrow).

Table 1. Embryonic stage correlated with the age (days) of embryos incubated at the FPT or MPT, and the degree of gonadal differentiation

Yntema stage	Approximate age (d)		Degree of gonadal differentiation
	26°C (MPT)	32°C (FPT)	
10	24 (n = 3)	14 (n = 3)	Germ cell migration
11	28 (n = 3)	17 (n = 3)	Germ cell migration
12	32 (n = 5)	18 (n = 3)	Germ cell migration
13	36 (n = 5)	19 (n = 3)	Germ cell migration
14	40 (n = 5)	22 (n = 4)	Germ cell migration/ Genital ridge
15	47 (n = 5)	25 (n = 4)	Genital ridge appearance
17	57 (n = 4)	31 (n = 4)	Differentiating gonads/ male
19	70 (n = 5)	37 (n = 5)	Differentiating gonads/ female
21	85 (n = 3)	47 (n = 3)	Differentiating gonads/ male, female
25	115 (n = 4)	92 (n = 4)	Differentiated

MPT = male producing temperature and FPT = female producing temperature.

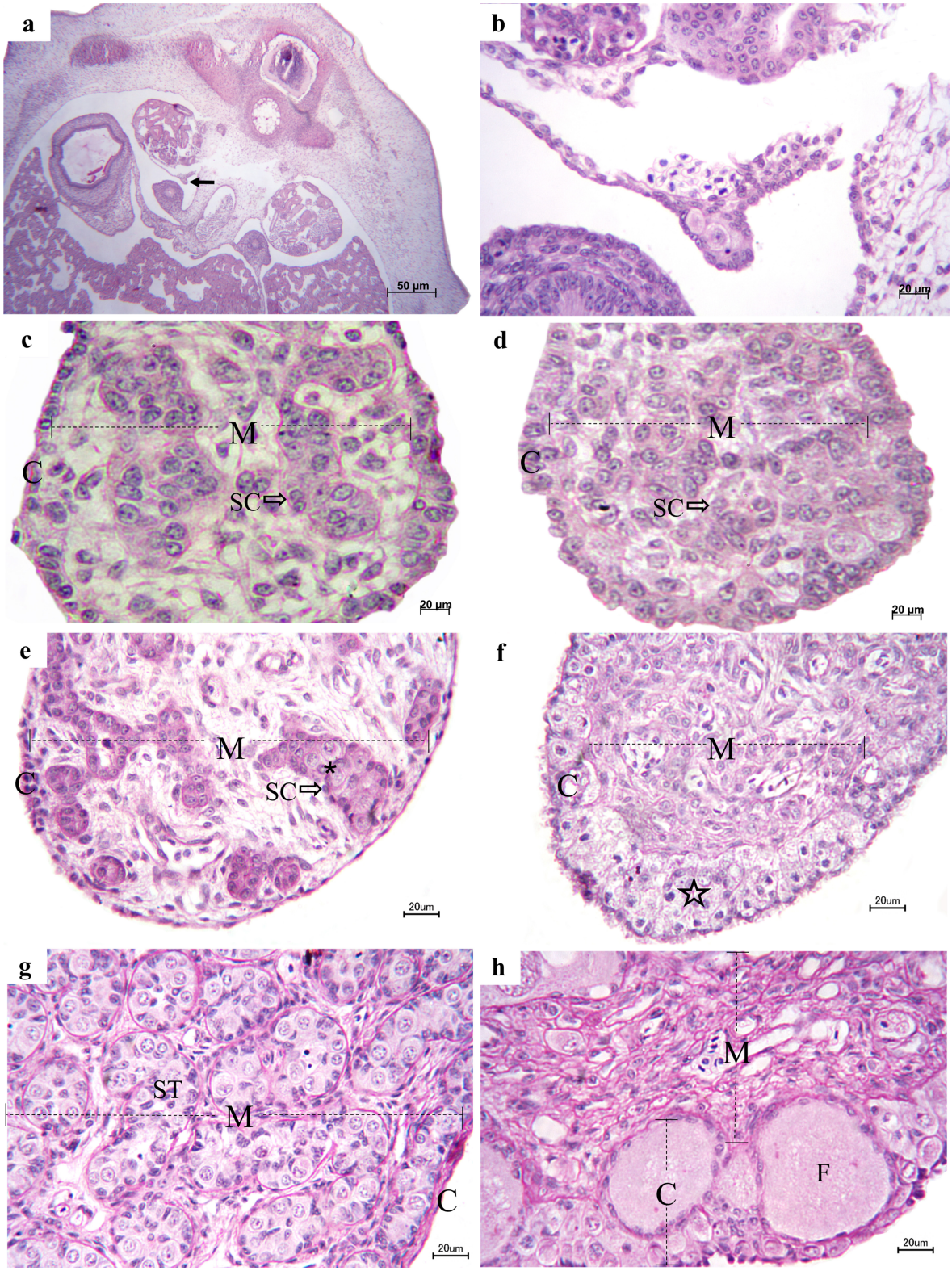

Fig. 2. Gonadal development during Yntema stages 15, 17, 19, and 25 when incubated at the MPT (26°C) or FPT (32°C). Location of the gonad at Yntema stages (a, b) 15 (black arrows), (c) 17 (MPT), (d) 17 (FPT), (e) 19 (MPT), (f) 19 (FPT), (g) 25 (MPT), and (h) 25 (FPT). C = cortical region or outer layer of tissue immediately below the epithelium; F = ovarian follicle; M = medullar region or the inner region of the gonad which is distinguishable from the outer region; SC = medullary sex cord; ST = seminiferous tubule; an asterisk indicates an area with germ cell division; a star indicates an area with cortical development.

revealed that the PGCs were still in the mesonephric duct and moved to a higher location than in stage 12 or 13 (Fig. 1e). Note that the PGCs of *M. macrocephala* are large with a round nucleus and pale cytoplasm, making them relatively easy to detect (Fig. 1f and g).

The genital ridge was apparent as an indifferent gonad starting from Yntema stage 14 (Fig. 2a and b), and could be divided into two zones: inner medulla and outer cortical layers. The indifferent gonad then developed into testis with medullary sex cords starting at stage 17 (day 31 and 57 at the FPT and MPT, respectively; Fig. 2c) and the level of germ cell division was distinctly higher in the medullary sex cords from stage 19 (day 70 at the MPT; Fig. 2e). In the female turtle, the gonad was identified as an undifferentiated gonad at stage 17 (day 31 at the FPT; Fig. 2d). Then the presumptive sex cords degenerated, followed by cortical development into the ovarian structure starting from stage 19 (day 37 at the FPT; Fig. 2f). Subsequently, testicular (day 115 at the MPT; Fig. 2g) and ovarian (day 92 at the FPT; Fig. 2h) development occurred independently and distinct sex organs became apparent at stage 25.

Detection of proliferating cells

The dynamics of germ cell proliferation were identified immunohistologically using PCNA staining. There were essentially no positive PCNA reactions in the indifferent gonad from embryos reared at the MPT (stage 15; Fig. 3a), but germ cell proliferation was found in the medullar at stage 17 (Fig. 3b). Afterwards, testicular development was strongly PCNA positive at stages 19 (Fig. 3c) and 25 (Fig. 3d). The cell proliferation (as PCNA positive staining) in embryos reared at the FPT was not detected in the indifferent gonad (stage 15; Fig. 4a) or at stage 17 (Fig. 4b), but it was found in the cortex at stage 19 (Fig. 4c), and weak PCNA signals were identified at stage 25 (Fig. 4d).

Detection of apoptotic cells

The TUNEL assay revealed no evidence of apoptosis at stages 15 (Fig. 5a) or 17 (Fig. 5b) in the embryos reared at the MPT, but germ cell apoptosis (strong TUNEL staining) was found in the cortex at stages 19 (Fig. 5c) and 25 (Fig. 5d). In embryos reared at the FPT, there was also no evidence of apoptosis at stages 15 (Fig. 6a) or 17 (Fig. 6b), but strong TUNEL signals were found in the medullar sex cord at stage 19 (Fig. 6c) and weak signals were found at stage 25 (Fig. 6d).

Strong cell proliferation and apoptosis were evident at stage 25 based on the immunohistochemical detection of proliferating (PCNA positive) and apoptotic (TUNEL positive) cells in the presumptive testis, whereas in the presumptive ovary both cell proliferation and apoptosis were weak at this stage.

DISCUSSION

An increase in the average global temperature can change both the physical and biological environment and affect the survival of organisms. Organisms that cannot adapt well to the new environment will decline and may become extinct (Walther et al. 2002). In many reptiles, gonadal differentiation is controlled by TSD. In most freshwater turtles, the sexual differentiation of gonads is influenced by the incubation temperature of the egg during the critical TSP of embryonic development (Valenzuela 2004). A previous histological analysis suggested that *M. macrocephala* undergoes TSD (Pewphong et al. 2016), so this research aimed to examine the chronology of gonadal development at the presumed MPT and FPT.

Prior studies have been conducted on the chronology of gonadal development in turtles using light microscopy (Ewert 1985), electron microscopy (Wibbels et al. 1991), and gene expression and synthesis (Ramsey et al. 2007). This study evaluated the germ cell migration of *M. macrocephala* from the extra-embryonic area to the genital ridge (Ewert 1985), since the PGCs in turtle species are initially located in the lateral posterior region before moving to the gut and dorsal mesentery, then the gonad (Bachvarova et al. 2009). This is similar to other vertebrates, in which the PGCs originate outside the embryo and later migrate to the genital ridge (Gilbert and Barresi 2016). Note that the PGCs of reptiles are large, spherical, and PAS positive and have a distinct nucleolus (Raynaud and Pieau 1985), making them relatively easy to study.

An understanding of the chronology of gonadal differentiation is essential for designing studies on how temperature affects the development and physiology of turtles, especially endangered ones (Wibbels et al. 1991; Rafferty and Reina 2014). A similar chronology of gonadal differentiation in *M. macrocephala* was inferred from the freshwater turtle *Emys orbicularis*, in which testis development was distinguishable at stage 17 and ovary development at stage 19 (Pieau et al. 1998).

In this study, the male turtle gonads at Yntema stage 17 incubated at the MPT were PCNA positive (proliferating cells) in the medullary sex cords, and TUNEL positive (apoptotic) germ cells were observed in the cortex, especially at Yntema stage 25. In the female turtle (incubated at the FPT), the presumptive ovary was strongly and weakly PCNA positive in the

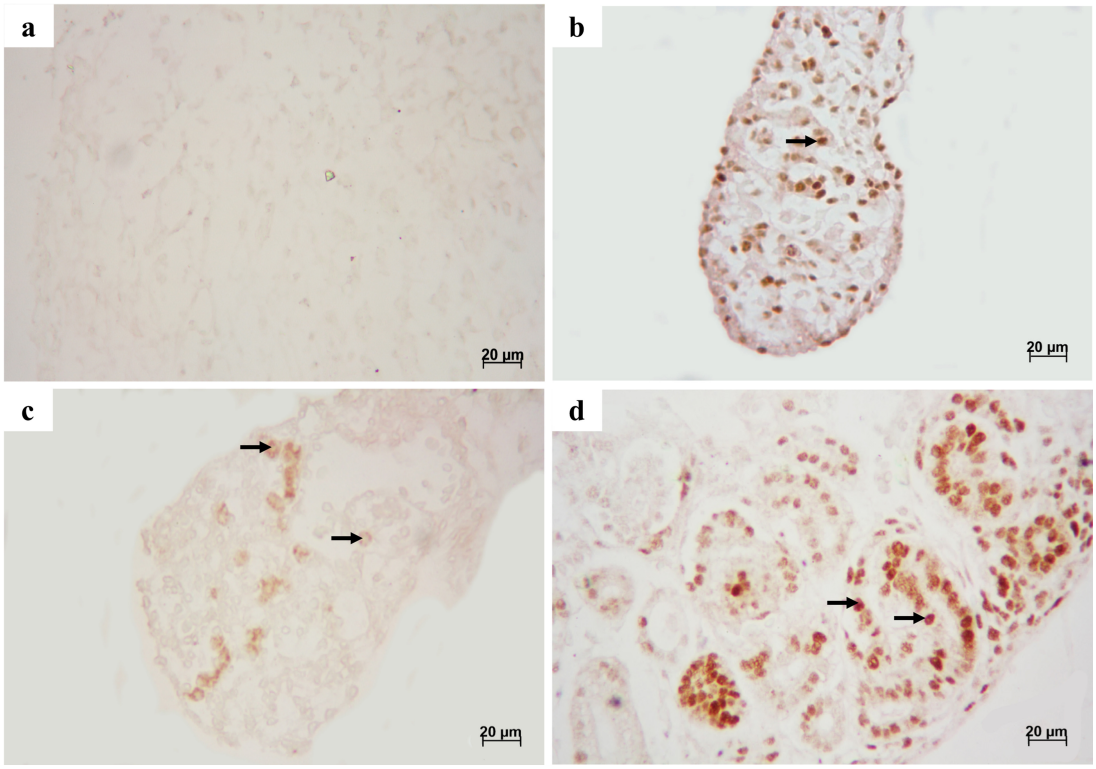

Fig. 3. Immunostaining of proliferating cells in the presumed male gonad of the *M. macrocephala* embryo at Yntema stages (b) 17, (c) 19, and (d) 25. Black arrows indicate PCNA-positive cells.

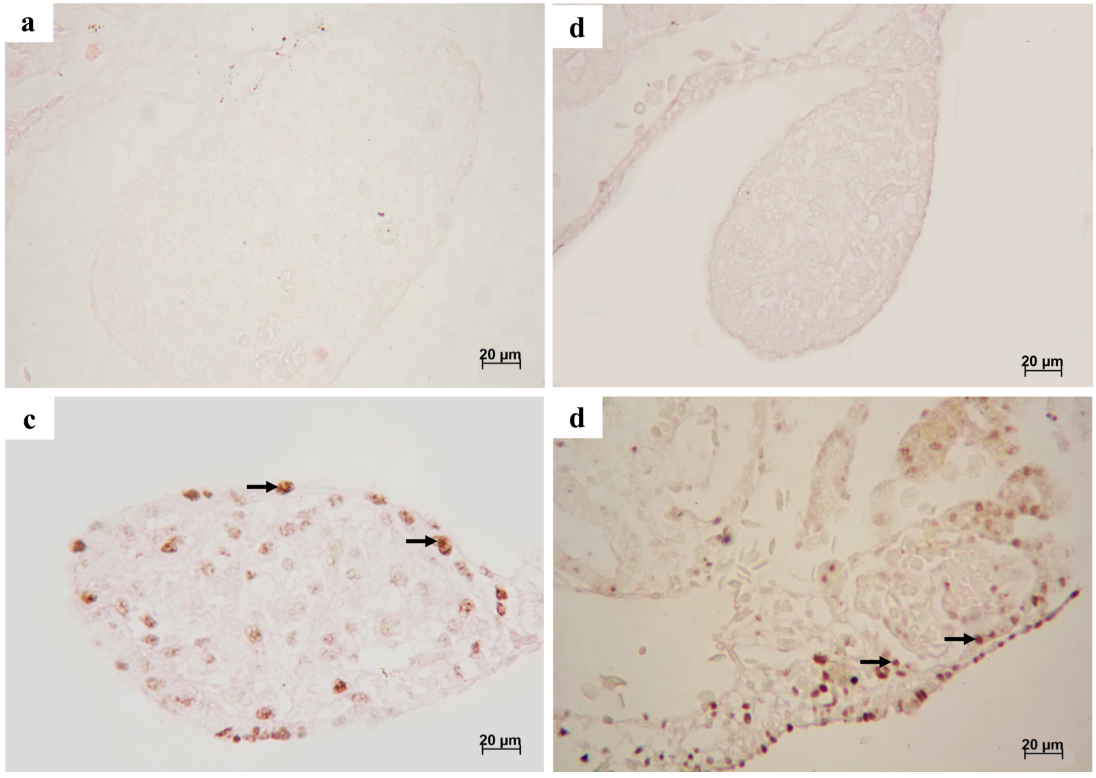

Fig. 4. Immunostaining of proliferating cells in the presumed female gonad of the *M. macrocephala* embryo at Yntema stages (a) 15, (b) 17, (c) 19, and (d) 25. Black arrows indicate PCNA-positive cells.

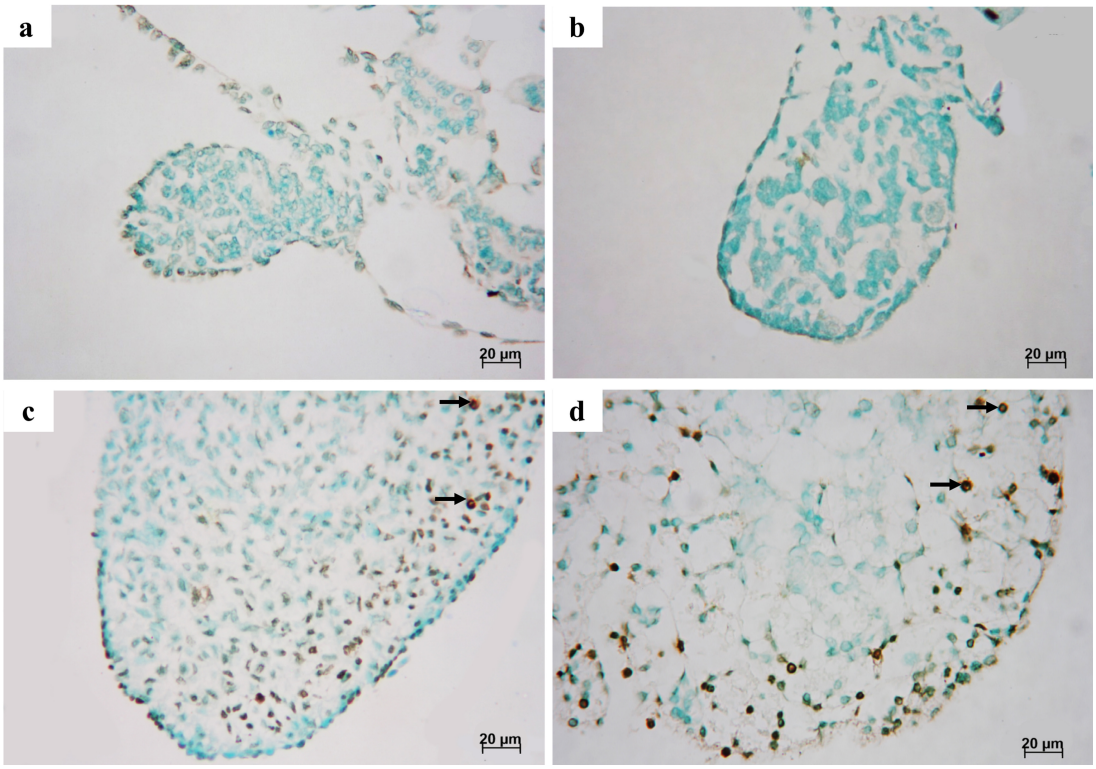

Fig. 5. Immunostaining of apoptotic cells in the presumed male gonad of the *M. macrocephala* embryo at Yntema stages (a) 15, (b) 17, (c) 19, and (d) 25. Black arrows indicate PCNA-positive cells.

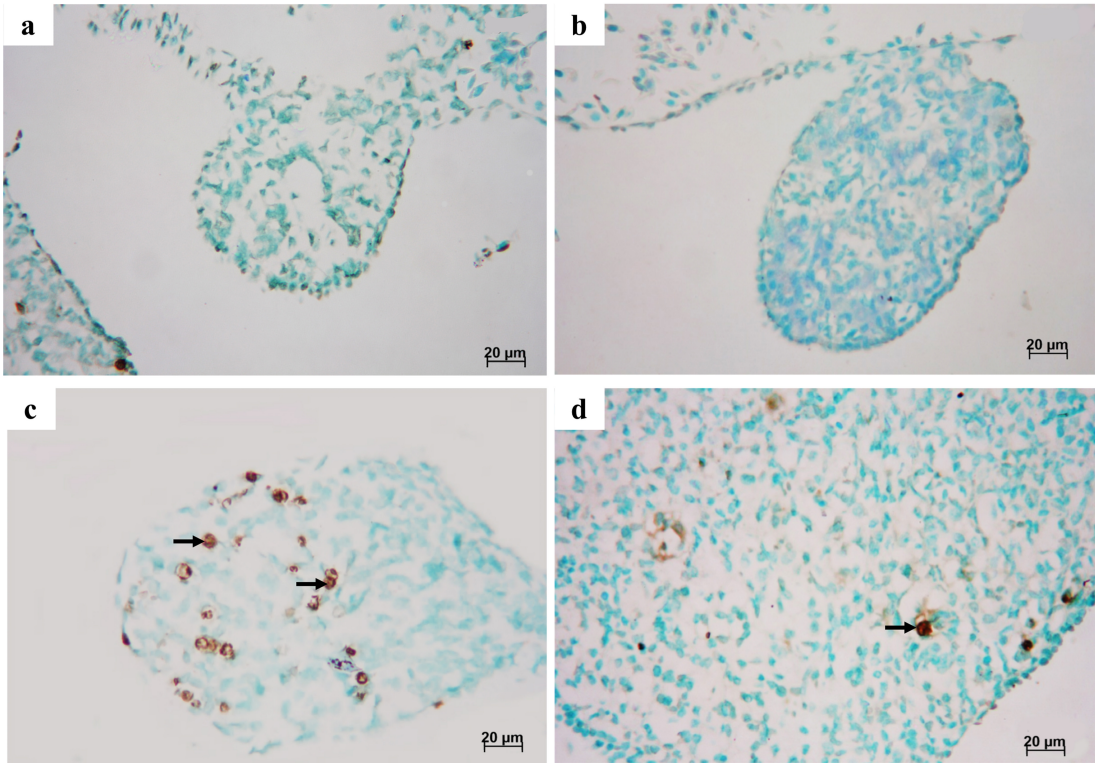

Fig. 6. Immunostaining of apoptotic cells in the presumed female gonad of the *M. macrocephala* embryo at Yntema stages (a) 15, (b) 17, (c) 19, and (d) 25. Black arrows indicate PCNA-positive cells.

cortex at Yntema stages 19 and 25, respectively, while strongly and weakly TUNEL positive germ cells were expressed in the medullar sex cord at Yntema stages 19 and 25, respectively. However, in the red-eared slider turtle, the sexual fate of males became fixed prior to that of females (Mork et al. 2014). The faster differentiation of male gonads than female gonads is well exhibited in vertebrates (Ramsey et al. 2007), and the sexual differentiation of gonads is associated with the growth rate (morphological stage) of embryonic development (Deeming and Ferguson 1988).

Based on sex determination in vertebrates, it is reasonable to suggest that sex factors control gonadal differentiation (Trukhina et al. 2013). Such sex factors in reptiles likely include sex steroids (Crews 1996), steroidogenic enzymes (*e.g.*, aromatase; Matsumoto and Crews 2012), and even heat shock proteins (Kohno et al. 2010). Using molecular techniques, Matsumoto et al. (2013) found that temperature may influence sex determination in freshwater turtles through epigenetic control of gene expression, including DNA methylation and histone modification (Matsumoto et al. 2016). In further studies, it would be interesting to evaluate the gonadal development of *M. macrocephala* using both hormonal and molecular approaches like those mentioned in the aforementioned studies, as well as other assays including functional assays (Schroeder et al. 2016) and genome-wide transcriptomic analyses (Liu et al. 2019).

CONCLUSIONS

The chronology of gonadal development in *M. macrocephala* can be split into four main gonadal development stages: germ cell migration, genital ridge appearance, testicular formation, and ovarian formation. The PGCs arise outside the genital ridge and from Yntema stages 10 to 14, and they can be observed migrating from the yolk sac into first the connective tissue of the hindgut, then the mesonephric duct, and finally the adjacent genital ridge. The genital ridge was apparent as an indifferent gonad starting from Yntema stage 14, and could be divided into the two zones of inner medulla and outer cortical layers. This gonad started developing into the testis with medullary sex cords at Yntema stage 17. In the female turtle, these sex cords then degenerated, followed by cortical development into an ovarian structure from Yntema stage 19. Subsequently, testicular and ovarian development occurred independently in male and female embryos, respectively, and distinct sex organs were apparent at Yntema stage 25.

These results are summarized in figure 7 as a chronological diagram of the developmental stages in embryos of *M. macrocephala*. In addition, the

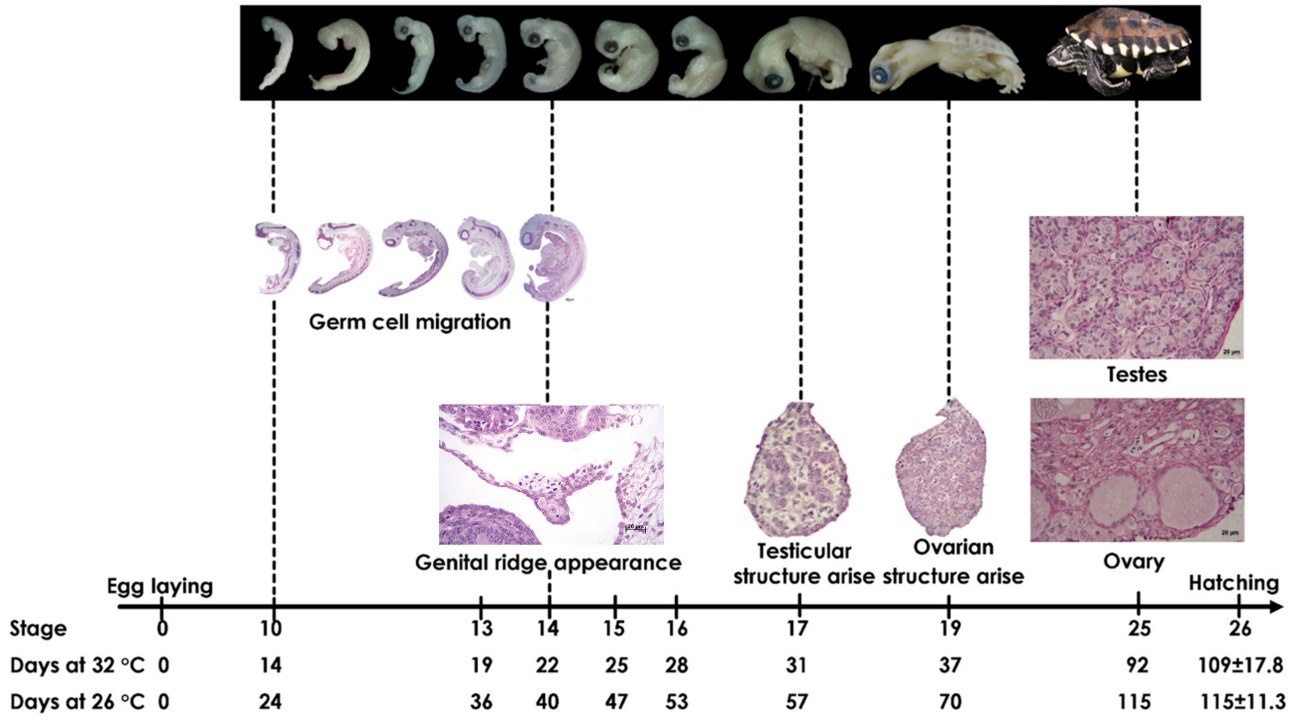

Fig. 7. Summary diagram of the chronology of developmental stages in embryos of the snail-eating turtle *M. macrocephala*.

results suggest that testicular and ovarian development involved proliferation and apoptosis at different stages. The presumptive testis showed germ cell proliferation in the medulla at Yntema stages 17, 19, and 25, and germ cell apoptosis in the cortex at Yntema stages 19 and 25. The presumptive ovary showed germ cell proliferation in the cortex at Yntema stages 19 and 25, and germ cell apoptosis in the medulla at Yntema stages 19 and 25. This outline of gonadal development could be applied to validate the use of this species as an animal model for research in developmental biology.

Acknowledgment: We thank Mr. Somyos Sooksumran for his aid collecting eggs, Dr. Robert J. D. Butcher (Research Clinic, Chulalongkorn University) and Noah Last (Third Draft Editing) for editing the English language of this manuscript, and members of the BioSentinel Laboratory at Chulalongkorn University for their assistance in the field. This research was supported by the 90th Anniversary of Chulalongkorn University Fund (Ratchadaphiseksomphot Endowment Fund). We also fully acknowledge an educational grant from the Human Resource Development in Science Project (Science Achievement Scholarship of Thailand) to RP.

Authors' contributions: All authors participated in designing the study and revising the manuscript. RP performed the field and laboratory works and wrote the draft manuscript. JK and NK supervised with the histological and immunohistochemical techniques and revised the manuscript. All authors read and approved the final manuscript.

Competing interests: RP, JK, and NK declare that they have no conflict of interests.

Availability of data and materials: The supporting data will be provided by the corresponding author upon request.

Consent for publication: Not applicable.

Ethics approval consent to participate: Animal procedures in this project were conducted in accordance with the Wild and Protected Animal Act of Thailand (1992) and were approved by the Department of Fisheries, Ministry of Agriculture and Cooperatives (Permit Number 17/2559) and the Chulalongkorn University Animal Care and Use Committee (Protocol Review Number 1723005).

REFERENCES

Bachvarova RF, Crother BI, Manova K, Chatfield J, Shoemaker CM, Crews DP, Johnson AD. 2009. Expression of *Dazl* and *Vasa* in turtle embryos and ovaries: evidence for inductive specification of germ cells. Evol Dev **11:**525–534. doi:10.1111/j.1525-142X.2009.00360.x.

Chan-ard T, Parr JWK, Nabhitabhata J. 2015. A field guide to the reptiles of Thailand. Oxford University Press, pp. 18–45.

Crews D. 1996. Temperature-dependent sex determination: the interplay of steroid hormones and temperature. Zool Sci **13:**1–13. doi:10.2108/zsj.13.1.

Deeming DC, Ferguson MWJ. 1988. Environmental regulation of sex determination of sex determination in reptiles. Philos Tran R Soc Lon B Sci **322:**19–39. doi:10.1098/rstb.1988.0111.

Ewert MA. 1985. Embryology of turtles. *In*: Gans C, Billet F, Maderson PFA (eds) Biology of the reptilia, vol 14: Development A. John Wiley & Sons, New York, pp. 75–267.

Fujimoto T, Ukeshima A, Miyayama Y, Horio F, Ninomiya E. 1979. Observations of primordial germ cells in the turtle embryo (*Caretta caretta*): light and electron microscopic studies. Dev Growth Differ **21:**3–10. doi:10.1111/j.1440-169X.1979.00003.x.

Gavrieli Y, Sherman Y, Ben-Sasson SA. 1992. Identification of programmed cell death *in situ* via specific labeling of nuclear DNA fragmentation. J Cell Biol **119:**493–501. doi:10.1083/jcb.119.3.493.

Gilbert SF, Barresi MJF. 2016. Developmental biology, 6th edition. Sunderland, Massachusetts, USA. Available from: https://www.ncbi.nlm.nih.gov/books/NBK9983/. Accessed 01 February 2019.

Girondot M, Kaska Y. 2014. A model to predict the thermal reaction norm for the embryo growth rate from field data. J Therm Biol **45:**96–102. doi:10.1016/j.jtherbio.2014.08.005.

Girondot M, Monsinjon J, Guillon JM. 2018. Delimitation of the embryonic thermosensitive period for sex determination using an embryo growth model reveals a potential bias for sex ratio prediction in turtles. J Therm Biol **73:**32–40. doi:10.1016/j.jtherbio.2018.02.006.

Ihlow F, Vamberger M, Flecks M, Hartmann T, Cota M, Makchai S, Meewattana P, Dawson JE, Kheng L, Rödder D, Fritz U. 2016. Integrative taxonomy of Southeast Asian snail-eating turtles (Geoemydidae: *Malayemys*) reveals a new species and mitochondrial introgression. PLoS ONE **11:**e0153108. doi:10.1371/journal.pone.0153108.

Keithmaleesatti S. 2008. An assessment of the association of organochlorine pesticide contamination and reproductive effects on the snail-eating turtle (*Malayemys macrocephala*) in the lower Chao Phraya river basin, Thailand. PhD dissertation, Chulalongkorn University, Thailand.

Kelman Z. 1997. PCNA: structure, functions and interactions. Oncogene **14:**629–640. doi:10.1038/sj.onc.1200886.

Kohno S, Katsu Y, Urushitani H, Ohta Y, Iguchi T, Guillette Jr LJ. 2010. Potential contributions of heat shock proteins to temperature-dependent sex determination in the American alligator. Sex Dev **4:**73–87. doi:10.1159/000260374.

Kyrylkova K, Kyryachenko S, Leid M, Kioussi C. 2012. Detection of apoptosis by TUNEL assay. Methods Mol Biol **887:**41–47. doi:10.1007/978-1-61779-860-3_5.

Liu T, Han Y, Liu Y, Zhao H. 2019. Genome-wide identification and analysis of heat-shock proteins 70/110 to reveal their potential functions in Chinese soft-shelled turtle *Pelodiscus sinensis*. Ecol Evol **9:**6968–6985. doi:10.1002/ece3.5264.

Matsumoto Y, Buemio A, Chu R, Vafaee M, Crews D. 2013. Epigenetic control of gonadal aromatase (*cyp19a1*) in temperature-dependent sex determination of red-eared slider

turtles. PLoS ONE **8:**e63599. doi:10.1371/journal.pone.0063599.

Matsumoto Y, Crews D. 2012. Molecular mechanisms of temperature-dependent sex determination in the context of ecological developmental biology. Mol Cell Endocrinol **354:**103–110. doi:10.1016/j.mce.2011.10.012.

Matsumoto Y, Hannigan B, Crews D. 2016. Temperature shift alters DNA methylation and histone modification patterns in gonadal aromatase (*cyp19a1*) gene in species with temperature dependent sex determination. PLoS ONE **11:**e0167362. doi:10.1371/journal.pone.0167362.

Mork L, Czerwinski M, Capel B. 2014. Predetermination of sexual fate in a turtle with temperature-dependent sex determination. Dev Biol **386:**264–271. doi:10.1016/j.ydbio.2013.11.026.

Pewphong R. 2012. Effects of temperature on gonadal development of the snail-eating turtle *Malayemys macrocephala*. PhD dissertation, Chulalongkorn University, Thailand.

Pewphong R, Kitana N, Kitana J. 2016. Effect of temperature on development: the case of the Malayan snail-eating turtle *Malayemys macrocephala*. *In*: Das I, Tuen A (eds) Naturalists, explorers and field scientists in South-east Asia and Australasia. Topics in biodiversity and conservation, vol 15. Springer, Cham. doi:10.1007/978-3-319-26161-4_10.

Pieau C, Dorizzi M. 1981. Determination of temperature sensitive stage for sexual differentiation of the gonads in embryos of the turtle *Emys orbicularis*. J Morphol **170:**373–382. doi:10.1002/jmor.1051700308.

Pieau C, Dorizzi M, Richard-Mercier N. 1999. Temperature-dependent sex determination and gonadal differentiation in reptiles. Cell Mol Life Sci **55:**887–900. doi:10.1007/s000180050342.

Pieau C, Dorizzi M, Richard-Mercier 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. doi:10.1002/(SICI)1097-010X(19980801)281:5%3C400::AID-JEZ5%3E3.0.CO;2-S.

Pipatsawasdikul K, Voris HK, Thirakhupt K. 2010. Distribution of the big-headed turtle (*Platysternon megacephalum*, Gray 1831) in Thailand. Zool Stud **49:**640–650.

Rafferty AR, Reina RD. 2014. The influence of temperature on embryonic developmental arrest in marine and freshwater turtles. J Exp Mar Biol Ecol **450:**91–97. doi:10.1016/j.jembe.2013.10.018.

Ramsey M, Crews D. 2009. Steroid signaling and temperature-dependent sex determination-Reviewing the evidence for early action of estrogen during ovarian determination in turtles. Semin Cell Dev Biol **20:**283–292. doi:10.1016/j.semcdb.2008.10.004.

Ramsey M, Shoemaker C, Crews D. 2007. Gonadal expression of *Sf1* and aromatase during sex determination in the red-eared slider turtle (*Trachemys scripta*), a reptile with temperature-dependent sex determination. Diff **75:**978–991. doi:10.1111/j.1432-0436.2007.00182.x.

Raynaud A, Pieau C. 1985. Embryonic development of the genital system. *In*: Gans C, Billet F, Maderson PFA (ed) Biology of the reptilia. Wiley, New York, pp. 151–299.

Sánchez-Ospina AC, Rodríguez B, Ceballos CP. 2014. Histological description of the reproductive tract of male and female hatchlings of the magdalena river turtle (*Podocnemis lewyana*). Acta Biol Colomb **19:**427–435.

Schroeder AL, Metzger KJ, Miller A, Rhen T. 2016. A novel candidate gene for temperature-dependent sex determination in the common snapping turtle. Genetics **203:**557–571. doi:10.1534/genetics.115.182840.

Tokita M, Kuratani S. 2001. Normal embryonic stage of the Chinese softshelled turtle *Pelodiscus sinensis* (Trionychidae). Zool Sci **18:**705–715. doi:10.2108/zsj.18.705.

Trukhina AV, Lukina NA, Wackerow-Kouzova ND, Smirnov AF. 2013. The variety of vertebrate mechanisms of sex determination. BioMed Res Int **2013:**1–8. doi:10.1155/2013/587460.

Valenzuela N. 2004. Evolution and maintenance of temperature-dependent sex determination. *In*: Valenzuela N, Lance V (ed) Temperature dependent sex determination in vertebrates. Washington D.C., Smithsonian Books, pp. 131–147.

Walther GR, Post E, Convey P, Menze A, Parmesank C, Beebee TJC, Fromentin JM, Guldbergi OH, Bairlein F. 2002. Ecological responses to recent climate change. Nature **416:**389–395. doi:10.1038/416389a.

Wibbels T, Bull JJ, Crews D. 1991. Chronology and morphology of temperature-dependent sex determination. J Exp Zol **260:**371–381. doi:10.1002/jez.1402600311.

Wolf HK, Dittrich KL. 1992. Detection of proliferating cell nuclear antigen in diagnostic histopathology. J Histochem Cytochem **40:**1269–1273. doi:10.1177/40.9.1354677.

Yao HHC, DiNapoli L, Capel B. 2004. Cellular mechanisms of sex determination in the red-eared slider turtle, *Trachemys scripta*. Mech Develop **121:**1393–1401. doi:10.1016/j.mod.2004.06.001.

Yntema CL. 1968. A series of stage in the embryonic development of *Chelydra serpentina.* J Morphol **125:**219–252. doi:10.1002/jmor.1051250207.