ผลของอุณหภูมิต่อการเจริญของอวัยวะสร้างเซลล์สืบพันธุ์ของเต่านา *Malayemys macrocephala*

นางสาวรังษิมา ผิวผ่อง

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
สาขาวิชาสัตววิทยา ภาควิชาชีววิทยา
คณะวิทยาศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2555
ลิขสิทธิ์ของจุฬาลงกรณ์มหาวิทยาลัย

บทคัดย่อและแฟ้มข้อมูลฉบับเต็มของวิทยานิพนธ์ตั้งแต่ปีการศึกษา 2554 ที่ให้บริการในคลังปัญญาจุฬาฯ (CUIR)
เป็นแฟ้มข้อมูลของนิสิตเจ้าของวิทยานิพนธ์ที่ส่งผ่านทางบัณฑิตวิทยาลัย
The abstract and full text of theses from the academic year 2011 in Chulalongkorn University Intellectual Repository(CUIR)
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EFFECTS OF TEMPERATURE ON GONADAL DEVELOPMENT OF THE SNAIL-EATING TURTLE *MALAYEMYS MACROCEPHALA*

Miss Rangsima Pewphong

A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science Program in Zoology
Department of Biology
Faculty of Science
Chulalongkorn University
Academic Year 2012
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Thesis Title	EFFECTS OF TEMPERATURE ON GONADAL DEVELOPMENT OF THE SNAIL-EATING TURTLE *MALAYEMYS MACROCEPHALA*
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รังษิมา ผิวผ่อง: ผลของอุณหภูมิต่อการเจริญของอวัยวะสร้างเซลล์สืบพันธุ์ของเต่านา *Malayemys macrocephala* (EFFECS OF TEMPERATURE ON GONADAL DEVELOPMENT OF THE SNAIL-EATING TURTLE *MALAYEMYS MACROCEPHALA*). อ.ที่ปรึกษาวิทยานิพนธ์หลัก: อ.ดร.นพดล กิตนะ, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: อ.ดร.จิรารัช กิตนะ, 129 หน้า.

การเพิ่มอุณหภูมิเฉลี่ยที่ทำให้โลกร้อนขึ้นอย่างต่อเนื่อง ทำให้สิ่งแวดล้อมทางกายภาพและชีวภาพเปลี่ยนแปลง และมีผลต่อการอยู่รอดของสิ่งมีชีวิต สัตว์เลื้อยคลานโดยเฉพาะเต่าน้ำจืดเป็นสิ่งมีชีวิตกลุ่มเสี่ยงต่อผลกระทบดังกล่าว เนื่องจากการเจริญของสัตว์กลุ่มนี้ได้รับอิทธิพลจากอุณหภูมิในขณะฟัก การศึกษาครั้งนี้มีวัตถุประสงค์เพื่อศึกษาผลของอุณหภูมิต่อการเจริญของร่างกายและอวัยวะสร้างเซลล์สืบพันธุ์ของเต่านา *Malayemys macrocephala* ซึ่งเป็นเต่าน้ำจืดพื้นเมืองชนิดที่พบมากที่สุดในประเทศไทย โดยเก็บตัวอย่างไข่เต่านาจากพื้นที่นาข้าวในอำเภอบางบาล จังหวัดพระนครศรีอยุธยา ในเดือนธันวาคม พ.ศ. 2554 ถึงเดือนกุมภาพันธ์ พ.ศ. 2555 ซึ่งจากการออกภาคสนาม 5 ครั้ง สามารถเก็บตัวอย่างไข่ได้ 712 ฟอง จาก 126 รัง เมื่อนำตัวอย่างมาศึกษาที่ห้องปฏิบัติการ โดยนำไข่เต่าเข้าฟักในตู้ควบคุมอุณหภูมิ ที่อุณหภูมิ 26, 29 และ 32 องศาเซลเซียส แล้วสุ่มเก็บตัวอย่างไข่จากการเพาะฟักทั้ง 3 อุณหภูมิจนกระทั่งออกเป็นตัว พบว่าเต่านาที่ได้จากการเพาะฟักทั้ง 3 อุณหภูมิ มีช่วงเวลาการออกเป็นตัวไม่แตกต่างกันอย่างมีนัยสำคัญ (115±11, 115±20 และ 109±18 วัน ตามลำดับ) เมื่อศึกษาการเจริญของเอ็มบริโอเต่าโดยเทียบลักษณะทางสัณฐานของเอ็มบริโอเต่านากับระยะการเจริญมาตรฐาน พบว่าเมื่อเปรียบเทียบรูปแบบการเติบโตของเอ็มบริโอจากระยะการเจริญและความยาวกระดองหลัง เต่านาที่ฟักที่อุณหภูมิสูง (32 องศาเซลเซียส) มีการเจริญเร็วกว่าที่อุณหภูมิต่ำ (26 องศาเซลเซียส) และอุณหภูมิกลาง (29 องศาเซลเซียส) อย่างมีนัยสำคัญ อย่างไรก็ตามเอ็มบริโอเต่าที่ฟักที่อุณหภูมิสูงมีการเจริญที่ผิดปกติมากกว่าช่วงอุณหภูมิอื่นอย่างมีนัยสำคัญ และเมื่อศึกษาการเจริญของอวัยวะสร้างเซลล์สืบพันธุ์จากลักษณะทางจุลกายวิภาคพบว่าอุณหภูมิในขณะฟักมีผลต่อการเปลี่ยนแปลงอวัยวะสร้างเซลล์สืบพันธุ์ให้เป็นอัณฑะหรือรังไข่ และมีผลต่อระดับการเจริญของอวัยวะสร้างเซลล์สืบพันธุ์อย่างมีนัยสำคัญ ซึ่งแสดงให้เห็นว่าเต่านามีการกำหนดเพศที่ขึ้นกับอุณหภูมิ ผลการศึกษาโดยรวมแสดงให้เห็นว่าอุณหภูมิเป็นปัจจัยสำคัญต่อการเจริญของร่างกายและอวัยวะสร้างเซลล์สืบพันธุ์ของเต่านา ซึ่งข้อมูลจากการศึกษานี้อาจนำมาใช้ประเมินผลกระทบจากแนวโน้มการเปลี่ยนแปลงอุณหภูมิโลก และเป็นแนวทางการพัฒนามาตรการลดผลกระทบที่อาจเกิดกับเต่าน้ำจืด เพื่อเป็นการอนุรักษ์ประชากรเต่าในธรรมชาติ

ภาควิชา ชีววิทยา ลายมือชื่อนิสิต .....

สาขาวิชา สัตววิทยา ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก .....

ปีการศึกษา 2555 ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์ร่วม .....

5372311723 : MAJOR ZOOLOGY
KEYWORDS : EGG INCUBATION / EMBRYONIC STAGE / FRESHWATER TURTLE / GROWTH PATTERN / SEX DETERMINATION / SEX RATIO

RANGSIMA PEWPHONG: EFFECS OF TEMPERATURE ON GONADAL DEVELOPMENT OF THE SNAIL-EATING TURTLE *MALAYEMYS MACROCEPHALA*. ADVISOR: NOPPADON KITANA, Ph.D.
Co-ADVISOR: JIRARACH KITANA, Ph.D., 129 pp.

Increase in average temperature that gradually warms the earth can change both physical and biological environments and affects survival of organisms. Reptiles, especially freshwater turtles, are considered as susceptible species since their development is dependent on incubating temperature. Current research thus aimed to examine effects of temperature on somatic and gonadal development of the snail-eating turtle, *Malayemys macrocephala*, a native and the most common freshwater turtles in Thailand. Turtle eggs were collected from rice fields in Bang Ban district, Phra Nakhon Si Ayutthaya province from December 2011 to February 2012. Based on 5 field surveys, a total of 712 eggs from 126 clutches of *M. macrocephala* were collected and transported to a laboratory. Eggs were incubated in microprocessor-controlled incubators at three different temperatures (26°C, 29°C and 32°C) and randomly dissected on weekly basis until hatch. It was found that incubation period, time from beginning of incubation until hatch, was not significantly different among temperatures (115$\pm$11, 115$\pm$20 and 109$\pm$18 days, respectively). Development of *M. macrocephala* was studied in reference to the standard stages of turtle development based on morphological characters. Growth patterns as indicated by stage of development and carapace length were significantly different among temperatures. At high temperature (32 °C), turtle embryos grew significantly more rapid than low temperature (26 °C) and pivotal temperature (29°C). However, embryos incubated at high temperature had a significantly higher incidence of malformation than other temperatures. Histological analysis for gonadal development showed that incubating temperature could affect differentiation of gonads into ovaries or testes as well as degree of development of the gonad, suggesting that *M. macrocephala* exhibits a temperature-dependent sex determination. Overall, the results indicated that incubating temperature is an important variable affecting both somatic and gonadal development of *M. macrocephala*. The data from this study could be used to assess the impact of global trend of temperature change and potential mitigation measure to reduce this impact on the freshwater turtle in order to conserve their natural populations.

Department : Biology Student's Signature .....

Field of Study : Zoology Advisor's Signature .....

Academic Year : 2012 Co-advisor's Signature .....

ACKNOWLEDGEMENTS

I wish to express my deepest gratitude and thanks to many people who contributed and supported in many ways during my study.

To my advisor, Dr. Noppadon Kitana, for his suggestions, guidance, patience and encouragement throughout the study. I am grateful to my thesis co advisor, Dr. Jirarach Kitana, for her kind guidance and suggestions.

To my thesis committee, Assistant Professor Dr. Duangkhae Sitthicharoenchai, Associate Professor Dr. Kumthorn Thirakhupt and Assistant Professor Dr. Duangjai Boonkusol, for their time and valuable discussion and suggestions.

I am thankful to Mr. Somyos Sooksuran, for his helps in turtle egg collection in the field, Dr. Ratchata Phochayavanich for his help in field work and suggestion on statistical analysis. I am also thankful to Dr. Thongchai Ngamprasertwong and Dr. Pongchai Dumrongrojwatthana for their advices on GIS map of Thailand.

Special thank is given to Mr. Tongchai Thitiphuree, for his suggestions in histological analysis. I am also thankful to Mr. Rachata Maneein for his suggestions in photography technique.

I would like to thank members of the BioSentinel Laboratory, Department of Biology, Faculty of Science, Chulalongkorn University including Mr. Panupong Thammachoti, Miss Mukrekha Chiewchanchai, Miss Yupaporn Visoot, Mr. Khattapan Jantawongsri and Mr. Sarawoot Gomuttapong, for their helps in turtle egg collection in the field trips, lab work and other support during my study.

In addition, appreciation is also extended to the Department of Biology, Faculty of Science, Chulalongkorn University for financial supports and facilities. I also would like to acknowledge a scholarship from the Human Resource Development in Science Project (Science Achievement Scholarship of Thailand).

Finally, I would like to give special thanks to everybody in my family for their supports and understanding during my study.

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CHAPTER I

INTRODUCTION

According to the latest meteorological data (IPCC, 2007), global temperatures are expected to increase compared to the global surface temperature records dated back since 1850. The climate change has warmed 0.3 to 0.6 °C over last 100 years (IPCC, 1996), and it is now widely accepted that this warming trend will continue (Hoegh-Guldberg et al., 2007). Global warming can change both physical and biological environment and affects survival of organisms. Organisms that cannot adjust oneself to suit with the changed environment may die down and become extinct (Walther et al., 2002). Climate change has direct and indirect effects on species and ecosystems (Blaustein et al., 1995). The direct effects of temperature on the physiology of organisms are well evidence, and many mechanisms of action have been identified. However, some effects of temperature may present unanticipated challenges to preservation (Vogt and Bull, 1982).

Reptiles, especially freshwater turtles, are considered as susceptible species since their sex determination is dependent of incubating temperature (Valenzuela, 2004a). Temperature dependent sex determination or TSD in reptiles provides an ideal model system with which to test predictions concerning the biological significance of global temperature change (Janzen, 1994).

There are three patterns of TSD as categorized by sex ratios produced in response to environmental temperature (Deeming and Feguson, 1988). First, TSD Ia (also termed male-female or MF) is used when male animals are produced at low temperature and female animals are produced at high temperature. Second, TSD Ib (also termed female-male or FM) is used when female animals are produced at low

temperature and male animals are produced at high temperature. Third, TSD II (also termed female-male-female or FMF) is used when female animals are produced at low and high temperatures and male animals are produced at intermediate temperature (Valenzuela, 2004a). Temperature extremes in the environment as a result of global warming can produce extreme biases in offspring sex ratios in species with TSD (Girondot et al., 2004).

In freshwater turtle, incubating eggs at different temperature could affect the incubation period and pattern of development (Packard et al., 1987; Pieau and Dorizzi, 1981). Many reports showed that increased incubation temperature resulted in earlier hatching (Ewert, 1979; 1985). The incubation temperature is also an important variable affecting hatching rate and development of freshwater turtle (Deeming and Ferguson, 1991; Grant et al., 2003) and can determine sex ratio of a turtle population (Bull et al., 1990; Willingham, 2005). As a result, an ecological model predicted that climate change may exhibit long-term impact on freshwater turtle populations (Parrott and Logan, 2010).

In addition, population dynamics of the freshwater turtle could be affected by deformities of the turtle (Davy and Murphy, 2009). Although developmental abnormalities with varying severity are not uncommon in wild populations of freshwater turtles (Ewert, 1979; MacCulloch, 1981; Pavaliko, 1986; Bell et al., 2006), thermal conditions during incubation were shown to have significant effects on embryonic growth and development in turtles (Deeming and Ferguson, 1991). Incubation at temperatures below or above optimal thermal range for significant periods of time could result in embryonic malformation (Booth, 2006).

In Thailand, although several species of freshwater are present (Nutphand, 1979; Thirakhupt and van Dijk, 1994), an extent of their susceptibility to temperature change is unknown due to the lack of information on their nesting biology, development pattern, somatic and gonadal development. In this study, the snail-eating turtle, *Malayemys macrocephala*, a native species and the most common freshwater in Thailand, was used as an animal subject. With its limited distribution in the Chao Phraya river basin (Brophy, 2004), rice fields in central part of Thailand, especially in Phra Nakhon Si Ayutthaya, are regarded as important breeding ground of this turtle species.

Since the information on nesting biology of *M. macrocephala* in the lower Chao Phraya River basin at Bang Ban district, Phra Nakhon Si Ayutthaya province is still limited (Keithmaleesatti, 2008), a study on this aspect was initially carried out to provide baseline natural history information of this species. The information of interest include nesting season, nest characteristics and clutch size.

Next, to use this species for reproductive and developmental biology studies, standard developmental stage of *M. macrocephala* was examined and established. Development of *M. macrocephala* was studied in reference to the widely use stages of turtle development of *Chelydra serpentina* (Yntema, 1968) based on morphological characters. Since some characters of *C. serpentina* are not presented in other freshwater turtles, staging criteria of *Pelodiscus sinensis* (Tokita and Kuratani, 2001) were used as supplemented criteria.

Futhermore, to examine effect of incubation temperature on development of *M. macrocephala*, somatic development of the turtle was studied at three different temperatures (26 °C, 29 °C and 32 °C). Information on incubation duration,

percentage egg weight loss, relative hatchling weight, growth patterns and somatic development were compared among incubating temperature.

Finally, effect of incubation temperature on gonadal development of *M. macrocephala* was studied at three different temperatures (26 °C, 29 °C and 32 °C). Although previous studies on this species showed a temperature-dependent pattern of sex determination as determined by morphometric analysis (Keithmaleesatti, 2008), the current study aimed to study gonadal development based on microanatomy in order to confirm this temperature-dependent pattern of sex determination in this turtle species as well as provided prediction on the potential effects of increased regional temperature on this native freshwater turtle species.

This information could be used to assess the impact of global trend of temperature change on sex ratio of turtle population and potential mitigation measure to reduce this impact of temperature change on turtle population in order to conserve their natural populations.

Objectives

1. To examine nesting biology of *M. macrocephala* in the lower Chao Phraya River basin at Bang Ban district, Phra Nakhon Si Ayutthaya province, the central part of Thailand
2. To examine normal developmental stage of *M. macrocephala*
3. To study effect of temperature on somatic and gonadal development of *M. macrocephala*

CHAPTER II

LITERATURE REVIEW

2.1 Climate change

According to the current meteorological data from the Intergovernmental Panel on Climate change (IPCC), air temperatures have increased to the level not seen since climatic records began in 1850 (IPCC, 2007). Over the past 100 years, the global temperature average has increased by approximately 0.3 °C to 0.6 °C (Gates et al., 1992). The rate of change has varied, with warming occurring most rapidly during the periods 1925 to 1944 and 1978 to 1997 (Jones et al., 1999). Temperature change is widely accepted and this warming trend is expected to be continuous (Edward and Richardson, 2004; Richardson and Schoeman, 2004). Almost 80 percent of the extra warmth is likely to be absorbed by the oceans and will result in thermal extension, which could produce an 18 to 60 cm rise in the sea levels by 2100 (Meehl et al., 2005). Therefore, rise in the sea level has been directed toward predicting the effects of temperature shifts on biological interactions (Kareiva, 1993).

Climate change, a gradual increasing in temperature average on the earth, can change both physical and biological environments and affects survival of organism. An average increase of approximate 0.5 °C is important for many physiological and ecological systems. Organisms that cannot adjust oneself to suit with the modified environment may die down and become extinct (Andrewartha and Birch, 1954; Walther et al., 2002). The direct effects of temperature on physiology of organism are well documented and many mechanisms of action have been identified (Wieser, 1973; Wood and McDonald, 1996). Some effects of temperature may present unanticipated challenges to conservation. For example, the sex of animals with temperature

dependent sex determination (TSD) is determined by environmental temperature (Bull, 1980). Temperature extremes in the environment as a result of global warming can produce extreme biases in offspring sex ratios in species with TSD (Girondot et al., 2004). The ecological model predicted that climate change may affect the freshwater turtle, *Chrysemys picta*, and demonstrated long-term impact on turtle populations (Parrott and Logan, 2010).

2.2 Development of turtle

2.2.1 Oviposition and embryonic orientation

Turtle exhibits an oviparity mode of reproduction which can be described by a deposition of fertilized amniotic eggs (Cloudsley-Thompson, 1999). The amniotic egg of turtle is surrounded by a tough outer shell that protects the egg from predators, pathogens, damage, and drying (Browder et al., 1991), because of the general lack of parental care in turtles and in most reptiles (Janzen and Warner, 2009). All turtles lay translucent and whitish eggs with unpigmented eggshells. The eggs are buried in soil and the developing embryos infrequently change position during embryogenesis. The dominant pattern of embryonic orientation in turtle share some character with bird (Romanoff, 1960). The early embryo of chick and turtle lies perpendicular to the long axis of its egg (Meier, 1981).

2.2.2 Indicators of development

Reptilian embryonic development was generally described based on the body size (length and mass), incubation age (time span), and development of extra embryonic membranes (Lillie, 1952; Hall and Miyake, 1995). Development is

typically documented in “stage” described the embryonic characteristics based on morphology related to incubation time (Andrew, 2004).

The most widely used index of development is standard body length. The measurement is focused on the total length for early embryos, followed by crown-rump length (CRL) in older embryos, and carapace length (CL) in embryos nearing hatching. However, each of these measurements becomes distorted at the embryos become increasingly curved during growth (Ewert, 1985).

2.2.3 Normal stage of development of turtle

The description of embryonic development in this field was focused on the early embryonic stages with neurulation as a common point of reference because it is more often assessed in embryological studies than the earlier stages (Moffat, 1985). An embryonic developmental stages of reptilian embryos based on the description of morphological changes were reported in snapping turtle *Chelydra serpentina* (Yntema, 1968), western painted turtle *Chrysemys picta bellii* (Mahmoud et al., 1973), marine turtle *Lepidochelys olivacea* (Crastz, 1982), and Chinese softshelled turtle *Pelodiscus sinensis* (Tokita and Kuratani, 2001).

Yntema (1968) presented the use of a normal series of developmental stages for the snapping turtle (*Chelydra serpentina*) with description of 27 stages (stage 0 to 26). Developmental stages were described based on incubation time of development at a given continual temperature. External morphology was described in each stage. Stages 0 (freshly laid egg) to 3 cover presomite neurulation. Stages 4 to 11 cover the somite period through the first presentation of separate limb buds. This phase includes the beginning of organogenesis. Stages 12 to 21 include limb development

through formation of the claws and the major period of organogenesis. And stages 22 to 26 refer to advanced embryos and hatching stage.

Mahmoud et al. (1973) reported normal developmental stages of the western painted turtle (*Chrysemys picta bellii*) embryos with description of 23 stages. The total body length was measured in the first 8 stages and the crown-rump length was measured in stages 9 through 15. The carapace length was measured after stage 15 until hatch. Normal development stage is similar to description of *C. serpentina*, with an exception of the sketched illustration instead of photographed illustration.

Crastz (1982) described 31 developmental stages (C-1 at laying to C-31 at hatching) of the Pacific Ridley turtle (*Lepidochelys olivacea*) based on embryo morphology and measurement. Each stage is qualified further with a computed numerical "index of morphological development" (IMD) based on continuous summation of a large number of discernible character situation. The rate of change in IMD is occasionally relative to the corresponding rate of change in days.

Miller (1985) reported 31 developmental stages of *Chelonia mydas* that obtained from measurements of embryonic body including crown-rump length in younger embryos and carapace length in older embryos. These developmental stage criteria were generally described according to embryonic stage of *C. serpentina*, *C. picta* and *L. olivacea*.

Tokita and Kuratani (2001) described 23 developmental stages (stages 5 through 27) of the Chinese softshelled turtle (*Pelodiscus sinensis*) from the late neurula stage to the hatching stage based on chronology and the external morphology of embryos. However, the early developmental stages (stages 0 to 4) was not available for description..

Greenbaum and Carr (2002) described the developmental stages of the spiny softshell turtle (*Apalone spinifera*). The descriptions of staging criteria were available for stages 13 through 26. Description of *A. spinifera* development were followed Yntema's (1968) terminology in describing the frontal, mandibular, maxillary, and nasal processes of embryos. Although Yntema's (1968) staging series is widely used for turtle, *A. spinifera* lack some key morphological characters to their highly divergent morphology such as carapacial scutes and digit with claws per limb.

Greenbaum (2002) presented standard series of embryonic development stages for the Emydid turtle (*Trachemys scripta*) in the latter portion (stages 12 through 26). In general, developmental stages were based on forelimb and claw morphology criteria described in *C. serpentina* (Yntema, 1968). However, this research used description on some disparate morphology such as carapace scute and urogenital papilla as criteria of development stages.

Table 2.1: Normal stage of development of turtle in relation to *Chelydra serpentina* (Yntema, 1968)

Yntema (1968) ***C. serpentina***	**Mahmoud et al. (1973)** ***C. picta belli***	**Crastz (1982)** ***L. olivacea***	**Miller (1985)** ***C. mydas***	**Tokina & Kuratani (2001)** ***P. sinensis***	**Greenbaum & Carr (2002)** ***A. spinifera***
0	-	-	-	-	-
1	1	-	-	-	-
2	2	1	-	-	-
3	3	2	-	-	-
4	4	3	9-10	5	-
5	5	4-5	11	6	-
6	6	6	11-12	7	-
7	7	6	12-13	8	-
8	7	7	14	9	-
9	8	8	15	10	-
10	9	9	15	11	-

Table 2.1: Normal stage of development of turtle in relation to *Chelydra serpentina* (Yntema, 1968) (continued)

Yntema (1968) ***C. serpentina***	**Mahmoud et al. (1973)** ***C. picta belli***	**Crastz (1982)** ***L. olivacea***	**Miller (1985)** ***C. mydas***	**Tokina & Kuratani (2001)** ***P. sinensis***	**Greenbaum & Carr (2002)** ***A. spinifera***
11	10	9	15	12	11
12	11	10	16	12	12
13	12	11	17	13	13
14	13	12	18-19	14	14
15	14-15	13-14	20-21	15	15
16	16	15	22	16	16
17	17	16	23	17	17
18	18	17-18	24-25	18-19	18
19	19	19	26	20	19
20	20	20-25	27	21	20
21	21	26-28	28	22	21

Table 2.1: Normal stage of development of turtle in relation to *Chelydra serpentina* (Yntema, 1968) (continued)

Yntema (1968) ***C. serpentina***	**Mahmoud et al. (1973)** ***C. picta belli***	**Crastz (1982)** ***L. olivacea***	**Miller (1985)** ***C. mydas***	**Tokina & Kuratani (2001)** ***P. sinensis***	**Greenbaum & Carr (2002)** ***A. spinifera***
22	21	26-28	28	23	22-23
23	22	29	29	24	22-23
24	22	29	29	25	24
25	22	30	30	26	25
26	23	31	31	27	26

Note: * Greenbaum (2002) stages for *T. scripta* are equivalent to those of Yntema (1968).

2.3 Sex determination in reptile

In most classes of vertebrates (with the exception of fishes and reptiles), genotypic sex determination (GSD) is a typical mode of sex determination. In GSD, parental chromosome sets in zygote determines the direction of development of the undifferentiated gonad towards male or female animal (Mrosovsky et al., 1984).

Sex determination in most, if not all, reptiles are sensitive to incubation or environmental temperature during embryonic development and cannot be predicted by genotype since no heteromorphic sex chromosome is presented. This unique mechanism, term "temperature dependent sex determination" or TSD (Bull, 1983; Valenzuela et al., 2003). In turtle, temperature dependent sex determination (TSD) is the most common mode of environmental mechanisms of sex determination (Ewert and Nelson, 1991; Ewert et al., 1994).

2.3.1 Temperature-dependent sex determination (TSD)

Under TSD, the sex of individuals is indifference until determined by incubation temperature (Bull, 1983). There are three patterns of TSD as categorized by sex ratios produced in response to incubation or environmental temperature (Deeming and Feguson, 1988). Three patterns of TSD (Figure 2.1) include:

(I) TSD Ia (also termed male-female or MF): male animals are produce at low temperature and female animals are produced at high temperature.

(II) TSD Ib (also termed female-male or FM): female animals are produced at low temperature and male animals are produce at high temperature.

(III) TSD II (also termed female-male-female or FMF): female animals are produced at low and high temperatures and male animals are produced at intermediate temperature.

Three patterns of TSD tend to associate with the direction of sexual dimorphism in adult size (Ewert and Nelson, 1991). TSD Ia occurs mainly in species with adult female animals larger than adult male animals. On the contrary, TSD Ib occurs in species with adult male animals larger than adult female animals. Pattern TSDII occurs mainly in species with no distinct sexual size dimorphism in adult animals.

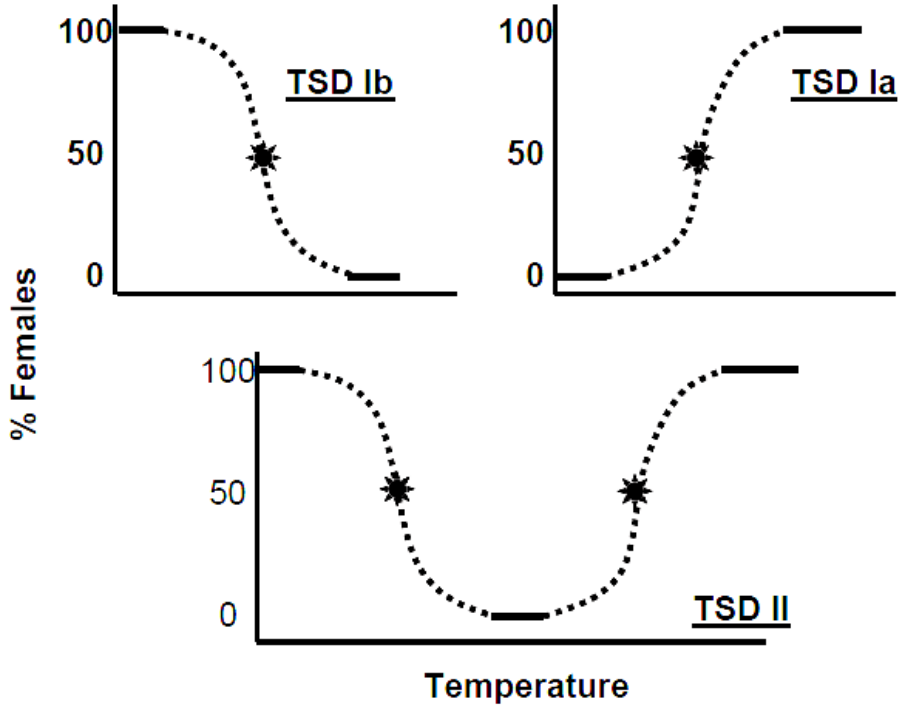


Figure 2.1 Patterns of TSD (TSD Ia, TSD Ib and TSD II) defined by the sex ratio produced as a function of constant incubation temperature. Dotted lines represent the transitional range(s). Stars denote the pivotal temperatures that correspond to constant temperatures that produce a population-wide 1:1 sex ratio (Valenzuela, 2004b).

2.3.2 Previous studies on TSD in turtle

Many species of turtle are reported to have TSD such as *Testudo graeca, Emys orbicularis* (Pieau, 1971), *Chelydra serpentina* (Yntema, 1978, 1979), *Pelodiscus sinensis* (Du and Ji, 2003) and *Chelodina rugosa* (Fordham et al., 2007). Incubating eggs at different temperature affects the incubation period and pattern of development of these species. Therefore, the incubation temperature is regarded as an important variable affecting hatching rate and development of freshwater turtle (Grant et al., 2003) and can determine sex ratios of a turtle population (Bull et al., 1990).

Previous studies aimed to examine pattern of TSD from 79 species of turtle showed that TSD patterns are different among species (Bull, 1980; Bull and Vogt, 1981; Ewert and Nelson, 1991). Sixty four species of turtle are known to have TSD, while 15 species are known to have GSD. Specific information of sex determination mechanism in turtle found in Southeast Asia is compiled and shown in Table 2.2.

Table 2.2: Summary of sex determination mechanism of turtle distributed in Southeast Asia

Species	Distribution	Mechanism	References
Family: Cheloniidae			
Chelonia mydas	Thailand, Vietnam and Cambodia	TSD	(Mrosovsky et al., 1984)
Eretmochelys imbricata	Thailand, Vietnam and Cambodia	TSD	(Mrosovsky et al., 1992)
Lepidochelys olivacea	Thailand, Myanmar and Malaysia	TSD	(McCoy et al., 1983)
Family: Geoemydidae			
Chinemys nigricans	Vietnam, China	TSD	(Ewert et al., 2004)
Mauremys mutica	Vietnam	TSD	(Ewert and Nelson, 1991)
Mauremys annamensis	Vietnam	TSD	(Ewert et al., 2004)
Melanochelys trijuga	Myanmar	TSD	(Ewert and Nelson, 1991)
Siebenrockiella crassiciollis	Vietnam, Cambodia, Myanmar, Malaysia,	GSD	(Carr and Bickhan, 1981)
Family: Emydidae			
Heosemys grandis	Thailand, Myanmar, Cambodia and Vietnam	TSD	(Ewert et al., 1994)
Trachemys scripta	Thailand, Taiwan and Cambodia	TSD	(Ewert and Nelson, 1991)

Note: TSD = temperature dependent sex determination, GSD = genotypic sex determination

2.4 Snail-eating turtle (*Malayemys macrocephala*)

2.4.1 Classification and distribution of *M. macrocephala*

Kingdom Animalia

Phylum Chordata

Class Reptilia

Order Chelonia

Family Geoemydidae

Genus *Malayemys*

Species *Malayemys macrocephala*

Common name: Snail-eating turtle

Thai name: Tao Na

The turtle in genus *Malayemys* (Lindholm, 1931) is a native species and the most common freshwater turtle in Thailand. Its range was recently restricted to a region extend in mainland of Southeast Asia such as in Cambodia (in all lowland areas), Laos (in lowlands of central and southern regions), Vietnam (in lowland of the south), Indonesia and Malaysia (Srinarumol, 1995).

2.4.2 Description of *Malayemys*

Brophy (2004) examined geographic variation and systematics of freshwater turtle populations in genus *Malayemys* from the Mekong River Basin and the Chao Phraya River Basins and reported that the traditionally conspecific *M. subtrijuga* turned out to represent the two distinct species in genus *Malayemys*. Turtle from the Mekong River Basin retain the name *M. subtrijuga* (Schlegel and Muller, 1844), whereas those from the Chao Phraya River basin are assigned the name *M. macrocephala* (Gray, 1859). Two characters were used as evidences to reveal

a clear pattern of geographic variation in genus *Malayemys* in cluding nasal stripe and infraorbital stripe. In *M. subtrijuga*, six or more than six nasal stripes are presented (Figure 2.2A) whereas four or less than four nasal stripes are presented in *M. macrocephala* (Figure 2.2B). The infraorbital stripe of *M. subtrijuga* is relatively narrow at the loreal seam (Figure 2.3A) whereas those of *M. macrocephala* is relatively wide at the loreal seam (Figure 2.3B).

M. subtrijuga is protected by CITES Appendix II and the Wild Animals Reservation and Protection Act B.E. 2535 of Thailand. International Union for Conservation of Nature and Natural Resources (IUCN) classified it as a vulnerable species (IUCN, 2007). However, *M. macrocephala* has not yet been protected by any national and international law (Clausnitzer et al., 2009).

Characteristics of *M. macrocephala* were described by Brophy (2004) as follows. The carapace is egg-shape with dark to light brown in color and a fine yellow line at the edge. The plastron is narrower than the carapace. The head is large and black with white stripe. The number of nasal stripe is four or less than four. The skin is gray and yellow. The limbs and the tail are gray to black.

Figure 2.2 Number of nasal stripe: (A) *M. subtrijuga* (arrow) (B) *M. macrocephala* (arrow).

Figure 2.3 Size of infraorbital stripe: (A) *M. subtrijuga* (arrow) (B) *M. macrocephala* (arrow).

2.4.3 Nesting biology and reproductive biology of *Malayemys*

Clutch size of *M. subtrijuga* (potentially included *M. macrocephala*) was reported as 5-10 eggs (Nutphand, 1979). It was reported that *M. subtrijuga* could lay more than one clutch of eggs per nesting season (Nutphand, 1979). Subsequent report on nesting biology of *M. subtrijuga* (potentially included *M. macrocephala*) at Phetchaburi province showed that the turtle could lay 3-6 eggs per clutch (Srinarumol, 1995). Eggs incubated in a moist mixture of sand and coconut-husk-fluff at ambient temperature (26-32 °C) resulted in incubation time of 97 to 292 days (Srinarumol, 1995).

With its limited distribution in the Chao Phraya river basin (Brophy, 2004), rice fields in central part of Thailand, especially in Phra Nakhon Si Ayutthaya, are regarded as important breeding ground of *M. macrocephala*. Previous records showed that an onset of nesting season of *M. macrocephala* in this area always occurred in November and last until April of the following year. Previous reports indicated that turtle nests were built as a u-shape hole with cover made from soil and plant materials (Keithmaleesatti, 2008). Clutch sizes of *M. macrocephala* were 3-10

eggs. Incubation of turtle eggs in moisted vermiculite in microprocessor-controlled incubator showed that the incubation period at 26-32 °C ranged from 82 to 186 days (Keithmaleesatti, 2008).

2.4.4 Characteristics of *Malayemys* eggs

Egg size, shape and number of eggs may be related to anatomy of the female oviparous reptiles, as well as the physiological ecology of the clutch in the nest (Packard and Packard, 1988). In reptiles, the typical shape of egg is spherical or ellipsoid. The snail-eating turtle egg is ellipsoid in shape like *Chelydra serpentina* and *Chrysemys picta* (Iverson and Ewert, 1991). Demension of *M. subtrijuga* eggs was 40-45 x 20-25 mm (Srinarumol, 1995). While egg dimension of *M. macrocephala* was 38 x 22 mm (Keithmaleesatti, 2008).

Figure 2.4 Morphology of *M. macrocephala* egg collected from rice field in Phra Nakhon Si Ayutthaya province.

CHAPTER III

NESTING BIOLOGY OF THE FRESHWATER TURTLE *MALAYEMYS MACROCEPHALA* AT PHRA NAKHON SI AYUTTHAYA PROVINCE, CENTRAL PART OF THAILAND

3.1 Introduction

The turtle in genus *Malayemys* (Lindholm, 1931) is a native species and the most common freshwater turtle in Thailand. Its range was recently restricted to a region extend in mainland of Southeast Asia such as in Cambodia (in all lowland areas), Laos (in lowlands of central and southern regions), Vietnam (in lowland of the south), Indonesia and Malaysia (Srinarumol, 1995). In the central part of Thailand, a large population of *Malayemys macrocephala*, a representative species of the genus *Malayemys*, can be found in rice fields at Phra Nakhon Si Ayutthaya province (Keithmaleesatti, 2008).

With its limited distribution in the Chao Phraya River basin (Brophy, 2004), rice fields in central part of Thailand, especially in Phra Nakhon Si Ayutthaya, are regarded as an important breeding ground of this turtle species. Previous records showed that an onset of nesting season of *M. macrocephala* in this area always occurred in November and last until April of the following year. Previous reports indicated that turtle nests were built as a u-shape hole with cover made from soil and plant materials (Keithmaleesatti, 2008). Clutch size of *Malayemys* turtle was reported at 5-10 eggs (Nutphand, 1979), 3-6 eggs (Srinarumol, 1995) and 3-9 eggs (Keithmaleesatti, 2008).

Current research aimed to examine nesting biology of *M. macrocephala* in the lower Chao Phraya River basin at Bang Ban district, Phra Nakhon Si Ayutthaya

province during 2011-2012. Parameters of interest include nesting season, nest characteristics and clutch size.

3.2 Materials and Methods

3.2.1 Study area and study period

Nesting activity and nesting sites were surveyed at the rice fields covering a 6 x 7.5 km^2 area in Bang Ban district, Phra Nakhon Si Ayutthaya province in central part of Thailand (UTM Zone 47P: 0653086-0659077 and 1583552-1591014; Figures 3.1). Rice is the predominant crop in this area. The area is an important breeding ground of the snail-eating turtle in central part of Thailand (Keithmaleesatti, 2008). Field surveys were conducted during November 2011 to February 2012 in nesting season of *M. macrocephala*.

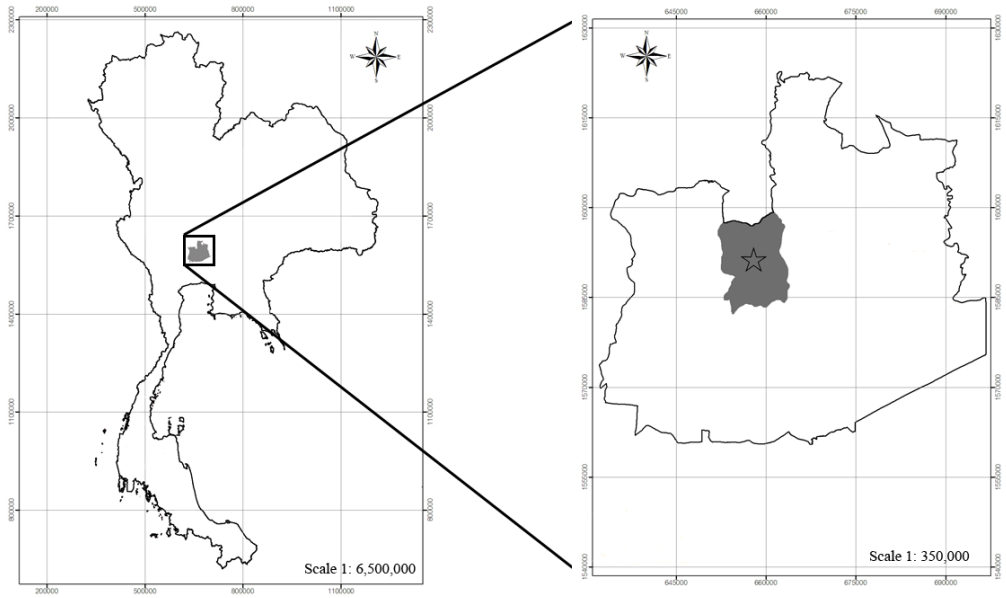


Figure 3.1: Study area (star) at Bang Ban district, Phra Nakhon Si Ayutthaya province, Thailand (UTM Zone 47P: 0653086-0659077 and 1583552-1591014).

3.2.2 Nesting activity and nest characteristics

Nest of the snail-eating turtle were located by visual encounter survey based on an expertise of the local farmer. Upon sighting, nest characteristics including dimension, temperature and clutch size were recorded. Nest dimension (width and depth) were measured by a measuring tape. Temperature of eggshell surface, nest soil and air were measured by an infrared thermometer. Number of eggs in each nest was counted. Eggs were stored in a styrofoam box (Figure 3.2) and transferred to the laboratory at the Department of Biology, Faculty of Science, Chulalongkorn University for further examination (Chapter IV-VI). The sampling method had been approved by the Chulalongkorn University Animal Care and Use Committee (Protocol Review No. 1323005).

3.2.3 Statistical analyses

Descriptive statistics (mean and standard error of the mean) were used to describe the data recorded in this study.

After checking for normality and homogeneity of variance, mean physical characters were compared among temperature in naturals nests (eggshell, nest soil and air) using one-way ANOVA followed by Bonferroni test.

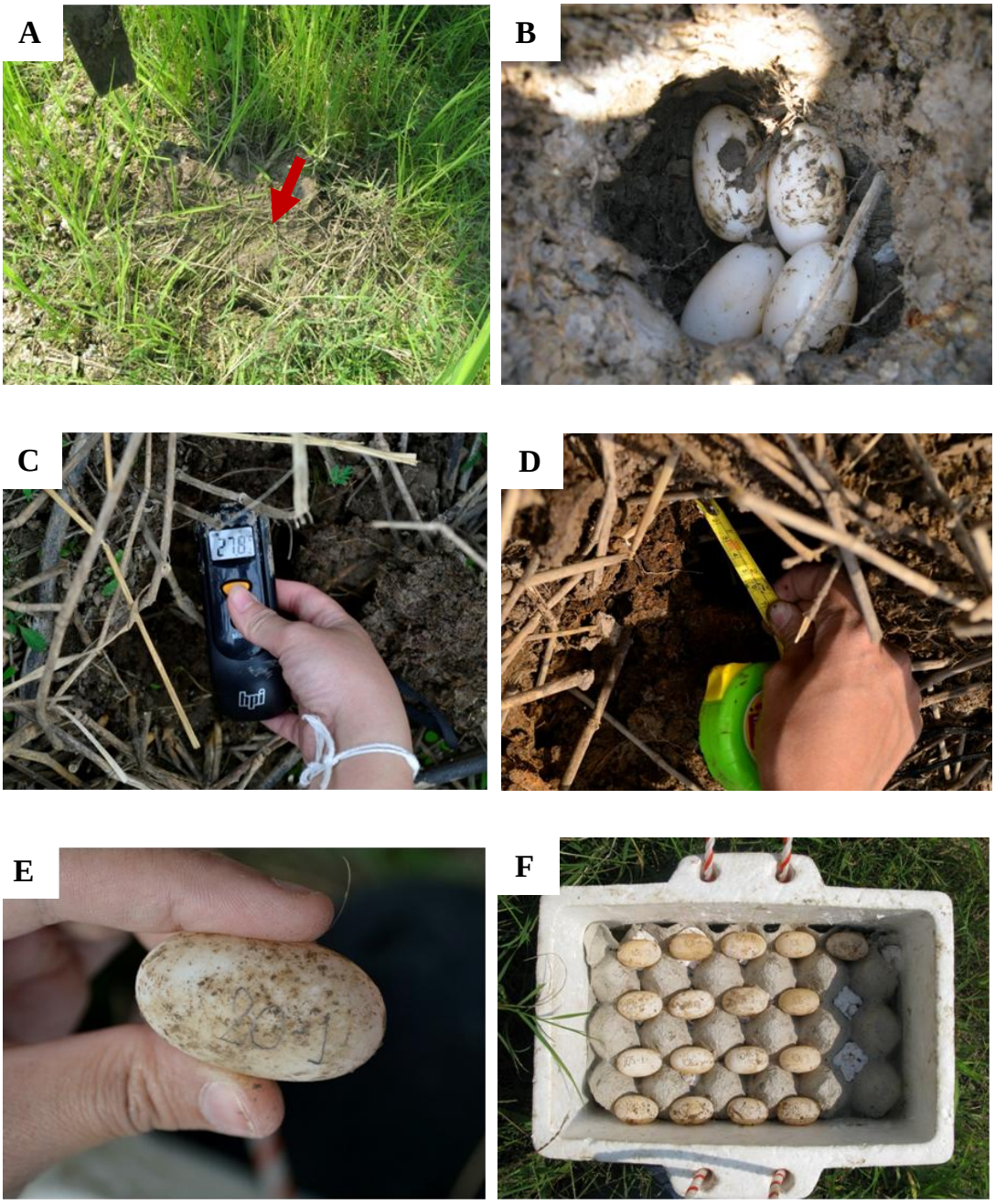


Figure 3.2: Survey for nesting activity of *M. macrocephala* at Bang Ban district, Phra Nakhon Si Ayutthaya province, Thailand: (A) nest location (an arrow) (B) nest excavation (C) temperature measurement (D) nest dimension measurement (E) egg labeling and (F) egg collection for further study

3.3 Results and Discussion

3.3.1 Nesting of *M. macrocephala* during 2011-2012

Field surveys in this study were conducted from November 2011 to February 2012 under supervision of a local farmer with more than 10 years of experience in turtle nest sighting. Although previous records (2005-2010) showed that an onset of nesting season of *M. macrocephala* in this area usually occurred in November (Keithmaleesatti, 2008), it is of interest to note that nesting activity was not found in November 2011 when most area was inundated as a result of the 2011 severe flooding in central part of Thailand. The earliest sighting of turtle nest was recorded in late December 2011 when dry area was available (Figure 3.3). Subsequent surveys in January-February 2012 revealed more sightings of the turtle nests in corresponding to available dry area (Figure 3.4 and 3.5).

Figure 3.3: Rice field at Bang Ban district, Phra Nakhon Si Ayutthaya province (47P 0655260 UTM 1588308) on 12 December 2011. Most area was inundated with patch of dry land dispersed throughout the area.

Figure 3.4: Rice field at Bang Ban district, Phra Nakhon Si Ayutthaya province (47P 0658833 UTM 1588506) on 26 January 2012. Water level in the rice field was minimal and the edge of the field was relatively dry.

Figure 3.5: Rice field at Bang Ban district, Phra Nakhon Si Ayutthaya province (47P 0653992 UTM 1588867) on 7 February 2012. Agricultural activity was resumed with rice covering approximately 40% of land. Dry land was available around the rice field.

3.3.2 *M. macrocephala* nest characters: Clutch size

Based on 5 field surveys, a total of 712 eggs from 126 clutches were recorded (Table 3.1). Compared with previous records of the closely related species, the clutch size found in this study (3-9 eggs) was similar to clutch size of *M. subtrijuga* (potentially included *M. macrocephala*) as reported by Nutphand (1979; 5-10 eggs) and Srinarumol (1995; 3-6 eggs).

Table 3.1: Clutch size of *M. macrocephala* at Bang Ban district, Phra Nakhon Si Ayutthaya province, Thailand during 2011-2012.

Date of survey	Number of clutch	Number of eggs	Clutch size (egg/nest)
25 Dec 2011	10	56	4-8
12 Jan 2012	26	142	3-9
26 Jan 2012	28	155	3-9
01 Feb 2012	30	185	4-9
07 Feb 2012	31	174	3-9
Total	**126**	**712**	**3-9**

3.3.3 *M. macrocephala* nest characters: Temperature and nest dimension

Average air temperature during the surveys was 30.72±4.65 °C. However, temperature inside the nest soil (28.47±3.80 °C) and temperature at eggshell (28.13±3.47 °C) were much lower (Table 3.2). There were significant differences in temperatures between air vs. nest soil and eggshell temperature. Compared to surveys in November 2005 and December 2006 (Keithmaleesatti, 2009), nest temperature in December 2011 (23.13 °C) was relatively lower than the past (30.4 °C). This could

be due to the difference in moisture content of the nesting ground as the ground was still wet in December 2011. However, the difference in eggshell temperature was minimal between years (22.88 °C vs. 24.6 °C). The incubation temperatures used in further study (26-32 °C; Chapter IV, V and VI) was within the range of temperature found in the natural nests.

Table 3.2: Physical characters of *M. macrocephala* nests at Bang Ban district, Phra Nakhon Si Ayutthaya province, Thailand during 2011-2012

Date of survey	Temperature (°C)			Nest dimension (cm)	
	Eggshell	Nest soil	Air	Width	Depth
25 Dec 2011	22.88±1.90	23.13±2.14	24.68±2.53	7.60±1.91	6.30±1.30
12 Jan 2012	25.35±2.49	25.24±2.69	27.28±3.88	6.48±1.07	6.63±1.13
26 Jan 2012	29.61±1.91	29.90±2.33	32.36±3.51	6.53±2.18	6.03±1.54
01 Feb 2012	27.42±2.02	27.99±2.46	30.74±3.26	6.50±1.58	7.07±1.17
07 Feb 2012	31.47±2.55	32.02±2.81	34.02±4.17	7.12±1.23	6.40±1.12
Mean±S.E.	**28.13±3.47[a]**	**28.47±3.80[a]**	**30.72±4.65[b]**	**6.74±1.62**	**6.52±1.29**

Remark: Significant difference between mean temperatures ($p<0.05$, one way ANOVA, Bonferroni test) is indicated by difference in superscript letters.

3.3.4 New record of eggshell malformation

Although turtle eggs of abnormal size and aggregation are not uncommon in marine turtle (Sahoo et al., 2009), eggshell malformation is quite rare in *M. macrocephala* and had never been reported in this area since 2005 (unpublished data). In this study, one clutch (out of 126 clutches surveyed) showed signs of eggshell malformation from mild to severe (Figure 3.6-3.7). In freshwater turtles that could lay more than one clutch per season (*Melanochelys trijuga* and *Rhinoclemmys areolata*), abnormally thick eggshell was believed to arise from oviductal retention of normally shelled eggs of one clutch that become reshelled during shelling of eggs in the next clutch (Ewert et al., 1984). Given the fact that *M. macrocephala* could lay more than one clutch of eggs per season (Nutphand, 1979), retention of eggs as a result of an absence in nesting site during the 2011 flooding of central part of Thailand could be one possible explanation for this eggshell abnormality.

Figure 3.6: Mild form of eggshell malformation in one clutch of *M. macrocephala* egg from Bang Ban district, Phra Nakhon Si Ayutthaya province during 2011-2012.

Figure 3.7: Severe form of eggshell malformation in one clutch of *M. macrocephala* egg from Bang Ban district, Phra Nakhon Si Ayutthaya province during 2011-2012.

3.4 Conclusion

Compared to previous report on nesting activity of *M. macrocephala* at Bang Ban district, Phra Nakhon Si Ayutthaya province, nesting season of *M. macrocephala* found in this study seemed to be delayed for almost a month, possibly due to the 2011 flooding in the central part of Thailand. Clutch size of the turtle (3-9 eggs) was similar to previous record of *Malayemys* turtles. Average nest temperatures ranged from 23.13 to 32.02 ℃ indicated potential exposure to wide range of temperature. Furthermore, new records of abnormality in turtle egg were documented in a clutch of turtle eggs found during this study period.

CHAPTER IV

A SERIES OF DEVELOPMENTAL STAGES OF THE SNAIL-EATING TURTLE *MALAYEMYS MACROCEPHALA* EMBRYO

4.1 Introduction

In Thailand, the snail-eating turtle (*Malayemys* sp.), is one of the native and the most common freshwater turtle (Nutphand, 1979; Thirakhupt and van Dijk, 1994). Compared to other freshwater turtle, baseline information about this species is readily available in regard to the natural history (Nutphand, 1979; Thirakhupt and van Dijk, 1994) and population biology (Srinarumol, 1995). Recently, *M. macrocephala* have been successfully used in environmental biology in both field conditions (Keithmaleesatti et al., 2009) and laboratory exposure (Kitana et al., 2008), suggesting a potential use of this species as an animal model for research in reproductive and developmental biology. Therefore, thorough investigation on basic developmental biology, especially on its normal stage of development, is needed for further utilization of this species.

Thorough investigation and description of embryonic development of turtle embryo was firstly established in the snapping turtle (*Chelydra serpentina)* by Yntema (1968). Morphological changes of *C. serpentina* were described from gastrula to hatching stages (stages 0 to 26) as follows. Stages 0 to 3 start at the freshly laid egg and cover presomite neurulation. Stages 4 to 11 cover the somite period through the first appearance of distinct limb buds. Stages 12 to 21 cover limb development through formation of the claws. Stages 22 to 26 refer to advanced embryos and hatchling (Yntema, 1968).

However, comparing most turtles with the staging criteria of *C. serpentina* is difficult because of disparate morphology and confused by the limited morphological description. Therefore, several other normal series have been described for various turtles including western painted turtle (*Chrysemys picta bellii*; Mahmoud et al., 1973), marine turtle (*Lepidochelys olivacea*; Crastz, 1982), green turtle (*Chelonia mydas*; Miller, 1985), Chinese softshelled turtle (*Pelodiscus sinensis*; Tokita and Kuratani, 2001), the spiny softshell turtle (*Apalone spinifera*; Greenbaum and Carr, 2002), and the slider turtle (*Trachemys scripta*; Greenbaum, 2002).

To establish a series of developmental stage for the snail-eating turtle, current research thus aimed to examine morphological changes during development of *M. macrocephala* embryo. This normal developmental stages could be applicable for further study on effect of temperature on somatic and gonadal development of the turtle (Chapter V and VI).

4.2 Materials and Methods

4.2.1 Study area and study period

Eggs of *M. macrocephala* were collected from rice fields in Bang Ban district, Phra Nakhon Si Ayutthaya province during December 2011 to February 2012. Based on 5 field surveys, a total of 712 eggs from 126 clutches were collected. Eggs were individually numbered, placed in a styrofoam box and transported to a laboratory at the Department of Biology, Faculty of Science, Chulalongkorn University. All portions of this research project involving turtle egg collection and animal subjects had been approved by the Chulalongkorn University Animal Care and Use Committee (Protocol Review Number 1323005).

4.2.2 Turtle egg incubation

In the laboratory, eggs were cleaned and weighed on an electronic balance (Tanita, accuracy 0.01 g). Eggs were randomly placed in plastic boxes containing moisted vermiculite (1 part vermiculite : 1 part distilled water, weight : volume). A total of 712 eggs were divided into three groups including 26 ℃ (n=237), 29 ℃ (n=237) and 32 ℃ (n=238). To control for consistency of incubating temperature, only eggs incubated at 29 ℃ were used for study on normal developmental stages. Eggs were kept in microprocessor-controlled incubators (Siam Incubators System, Bangkok, Thailand; Figure 4.1). Eggs were buried completely in the medium and the boxes were covered with plastic lids. A tray of water was placed inside each incubator to controlled relative humidity in excess of 80%. Temperature and relative humidity inside the box were monitored using a data logger (HAXO-8, LogTag Recorders, Auckland, New Zealand). Eggs were randomly sampled, weighed and dissected on weekly basis until hatch.

Figure 4.1: Egg incubation in a microprocessor-controlled incubator (Siam Incubators System, Bangkok, Thailand).

4.2.3 Staging and morphometry of turtle embryo

After dissection, developmental stage of turtle embryo was studied in reference to the widely use developmental stage of *Chelydra serpentina* (Yntema, 1968) and *Pelodiscus sinensis* (Tokita and Kuratani, 2001). Body length was measured by a vernier caliper (Mitutoyo, accuracy 0.01 mm) and used as an indicative of size of embryo. Crown-rump length (CRL) was measured at stage 3 to 14 and carapace length (CL) was measured at stage 15 to 26 (Figures 4.1). After measurement, embryos were subjected to euthanasia by injection with pentobarbital sodium at a dose of 600 mg/kg intraperitoneally. Embryos were fixed in Davidson's fixative for 24 hours and then preserved in 70% ethanol. Photographs of early stages 3 through 14 were taken with a digital camera connected to a microscope (Nikon AZ100). For embryonic stages 15 until stage 26, photographs were taken with a digital camera (Nikon D700).

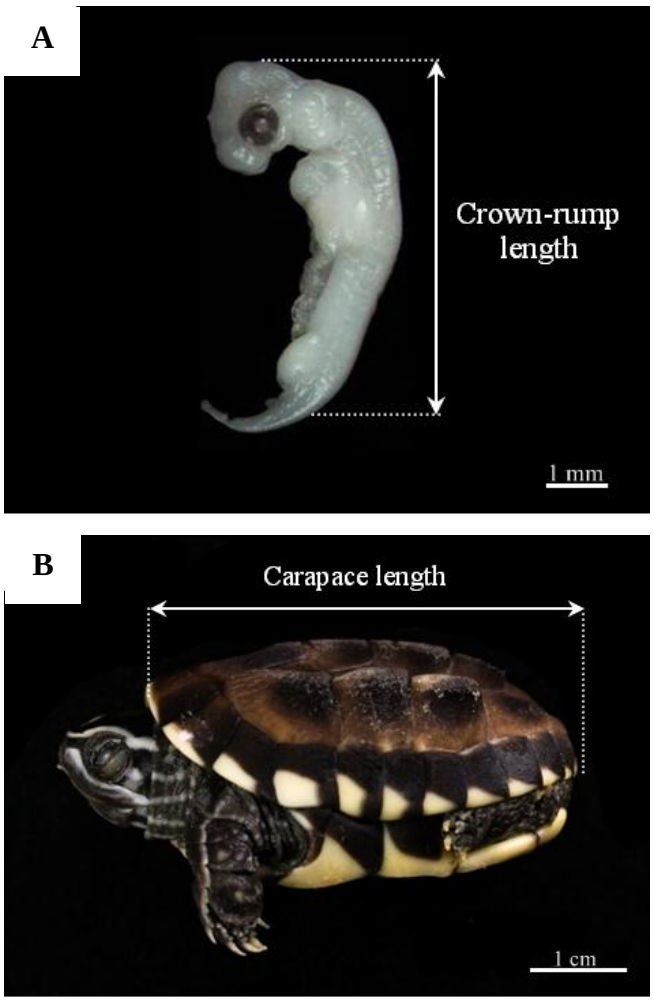


Figure 4.2: Measurement for body length as an indicative of size (A) crown-rump length (CRL) for embryonic stage 3 to 14 and (B) carapace length (CL) for embryonic stage 15 to 26.

4.2.3 Statistical analysis

Descriptive statistics (mean and standard error of the mean) were used to describe the morphological change during the course of development.

4.3 Results and Discussion

4.3.1 Staging of *M. macrocephala* embryo

In this study, the widely use stages of *Chelydra serpentina* development (Yntema, 1968) based on morphological characters and Yntema's terminology in describing the cranial structure, extra embryonic structures, body fold and limb of embryos were used as a reference. Since some characters of *C. serpentina* are not presented in other freshwater turtles, staging criteria of *Pelodiscus sinensis* (Tokita and Kuratani, 2001) were used as supplemented criteria (sensory organs, pharyngeal arches, and limbs). It was found that the earliest *M. macrocephala* embryo found in this study was at Yntema's stage 6. An absence of stages 0-5 embryo is possibly due to rapid development in the early stage of *M. macrocephala* as well as unmanageably small size of the embryo. After 24 weeks of incubation, 21 discrete stages (stages 6-26) of *M. macrocephala* embryos were found. In each stage, morphogenesis was substantially completed similar to previous study (Shine and Elphick, 2001). Detailed description of each developmental stage of *M. macrocephala* is listed as follows.

Stage 6 (Figure 4.3A)

Size: 3.57 mm (n=1, CRL)

Incubation period: 15 days at 29 °C

Cranial structures: Neural folds are opened extensively from anterior to posterior.

Extra-embryonic structures: Blood islands occur in the opaque area.

Body fold: The intestinal portal is at the region of the heart.

Stage 7 (Figure 4.3B)

Size: 4.23±0.29 mm (n=2, CRL)

Incubation period: 18±5 days at 29 °C

Cranial structures: Neural folds are remained. The anterior portion of head has moved the curve ventrally due to cephalic bend. The mandibular arch starts to form.

Extra-embryonic structures: Blood islands are remained and indicated in the opaque area.

Body fold: Lateral body fold is the limited area of the notochord.

Stage 8 (Figure 4.3C)

Size: 5.31 mm (n=1, CRL)

Incubation period: 19 days at 29 °C

Cranial structures: Neural folds form completely. The optic cyst is cup-shaped. Cervical flexure is visible.

Body fold: Heart is S-shape. The lateral body fold is visible.

Stage 9 (Figure 4.3D)

Size: 5.34±0.06 mm (n=2, CRL)

Incubation period: 29±6 days at 29 °C

Cranial structures: Increased cranial flexure has turned the head.

Body fold: The lateral body folds are arisen along the length of the embryo. The maxillary process is starting to form. The tail process is limited posteriorly.

Stage 10 (Figure 4.3E)

Size: 5.41 mm (n=1, CRL)

Incubation period: 29 days at 29 °C

Cranial structures: Neural folds are completely closed. The optic and nasal pits are visible.

Body fold: The posterior intestinal portal is visible.

Stage 11 (Figure 4.3F and Figure 4.6A)

Size: 5.09 mm (n=1, CRL)

Incubation period: 29 days at 29 °C

Cranial structures: Optic vesicles are clear and lack retina pigmentation. The pharyngeal slit is opened and slit is covered by the hyoid arch. The maxillary process extends toward the optic vesicles. The nasal pit has deepened.

Limbs: Both pairs of forelimb buds are obvious.

Flexures: Cervical flexure has increased in the bend.

Stage 12 (Figure 4.4A and Figure 4.6B)

Size: 5.63 mm (n=1, CRL)

Incubation period: 29 days at 29 ℃

Cranial structures: The optic part of retina is pigmented. The maxillary process extends as far as mandibular process. The nasal groove fold posterior beyond the margin of the lens.

Limbs: Forelimb bud has become thicker and longer.

Flexures: Tail has increased in the length.

Stage 13 (Figure 4.4B and Figure 4.6C)

Size: 6.72±0.12 mm (n=2, CRL)

Incubation period: 29±10 days at 29 ℃

Cranial structures: The retina has black pigmentation. The nasal groove fold posterior beyond the lower of the optic.

Limbs: The limb buds are longer than wide, forelimb buds and hindlimb buds are equal length. The digital plate is obvious in the forelimb.

Flexures: The cervical flexure has increased and the crown-rump length has decreased.

Stage 14 (Figure 4.4C and Figure 4.6D)

Size: 7.90±0.32 mm (n=4, CRL)

Incubation period: 29±6 days at 29 ℃

Cranial structures: The maxillary process and nasal groove are fused.

Limbs: Digit plate is obvious in the hindlimb.

Carapace: Body flexure is at maximum and carapace ridge has appeared along the trunk.

Stage 15 (Figure 4.4D and Figure 4.6E)

Size: 5.89±0.85 mm (n=2, CL)

Incubation period: 33±5 days at 29 ℃

Cranial structures: Surface of the neck has become directly due to extension of the flap of the hyoid arch.

Limbs: Digit plate is well formed.

Carapace: Carapace ridge is separate.

Stage 16 (Figure 4.4E and Figure 4.6F)

Size: 6.18 mm (n=1, CL)

Incubation period: 36 days at 29 ℃

Cranial structures: Lower jaw extends beyond the choroid.

Limbs: Interdigit groove and the digit ridge are formed on the digit plate.

Carapace: The carapace is clearly limited around perimeter.

Stage 17 (Figure 4.4F and Figure 4.6G)

Size: 7.59±0.75 mm (n=4, CL)

Incubation period: 36±6 days at 29 ℃

Cranial structures: Frontonasal process is obvious and the snout has extent to the anterior.

Limbs: Periphery of the digit plate is serrated. Digital ridges and interdigital grooves become obvious.

Carapace: Carapace has increased pigmentation. Pigmentation area has formed on the central of carapace.

Stage 18 (Figure 4.4G and Figure 4.6H)

Size: 9.24±0.62 mm (n=5, CL)

Incubation period: 44±3 days at 29 ℃

Cranial structures: Lower jaw is complete.

Limbs: Digital plate show clear digits with deep serrations and more spread. Spot of pigmentation has occurred on the limb.

Carapace: Carapace has increased pigmentation. Pigmentation area has formed on the central of carapace.

Stage 19 (Figure 4.5A and Figure 4.7A)

Size: 11.67±1.03 mm (n=4, CL)

Incubation period: 50±6 days at 29 °C

Cranial structures: Facial pigmentation has increased in its intensity. The lower eyelid is obvious.

Limbs: Digits become obvious with presence of the webs in between.

Carapace: Pigmentation of the mid-dorsal of the carapace has increased. The plastron margin is clearly formed.

Stage 20 (Figure 4.5B and Figure 4.7B)

Size: 14.52±0.72 mm (n=4, CL)

Incubation period: 53±4 days at 29 °C

Cranial structures: Lower eyelid still remains of the scleral papillae.

Limbs: Bases of the digits are lightly pigmented.

Carapace: Central and lateral of carapace are brown with pigment. The plastron is brown pigmented.

Stage 21 (Figure 4.5C and Figure 4.7C)

Size: 17.16±1.02 mm (n=5, CL)

Incubation period: 57±10 days at 29 ℃

Cranial structures: Scleral papillae have disappeared. A head with white stripes becomes visible and rows of cutaneous papillae are indicated on the dorsum of the neck.

Limbs: Bases of the digits are lightly pigmented.

Carapace: Pigmentation of the carapace is similar and number of the brown spots increases.

Stage 22 (Figure 4.5D and Figure 4.7D)

Size: 20.03±1.02 mm (n=4, CL)

Incubation period: 67±13 days at 29 ℃

Cranial structures: Lower eyelid covers most of the pupil in all specimens.

Limbs: Light colored claws are formed.

Carapace: The pigmentation of the carapace and neck rapidly increases.

Stage 23 (Figure 4.5E and Figure 4.7E)

Size: 24.24±1.50 mm (n=12, CL)

Incubation period: 61±9 days at 29 ℃

Cranial structures: Upper and lower eyelid are separated by the slit in all specimen.

Limbs: Pigmentation of the claw is heavy. The cutaneous folds are present over dorsum of the forearm.

Carapace: Pigmentation of the carapace is increase in brownish but not yet complete.

Stage 24 (Figure 4.5F and Figure 4.7F)

Size: 29.59±2.76 mm (n=18, CL)

Incubation period: 83±12 days at 29 ℃

Limbs: Cutaneous folds are more prominent. The claw is blunt and extents the length of its rudiment.

Carapace: Carapace has increase pigmentation.

Stage 25 (Figure 4.5G and Figure 4.7G)

Size: 31.22±2.18 mm (n=82, CL)

Incubation period: 129±25 days at 29 °C

Limbs: The ungual phalanx and claw are detached. The claw is longer, therefore, the distance between the apexes of the claw is slightly greater.

Carapace: The pigmentations of carapace and plastron of the embryo like that of hatching stage. The dorsal keel is reduced.

Stage 26 (Figure 4.5H and Figure 4.7H)

Size: 31.34±2.17 mm (n=26, CL)

Incubation period: 115±20 days at 29 °C

Limbs: Claw on the forelimb is translucent at its apex and pigment on the base of the claw is extensive and the claw is sharp.

Carapace: Pigmentation of the carapace has increased and coloration is brown with a light creamy peripheral border. The plastron is heavily pigmented and reaches along the seams of the scutes.

Hatching period: Immediately pipping and leaving of the shell.

Figure 4.3: Photographs of embryonic stages of *M. macrocephala* in lateral view A-F; (A) stage 6 (B) stage 7 (C) stage 8 (D) stage 9 (E) stage 10 (F) stage 11. Scale bar = 1 mm.

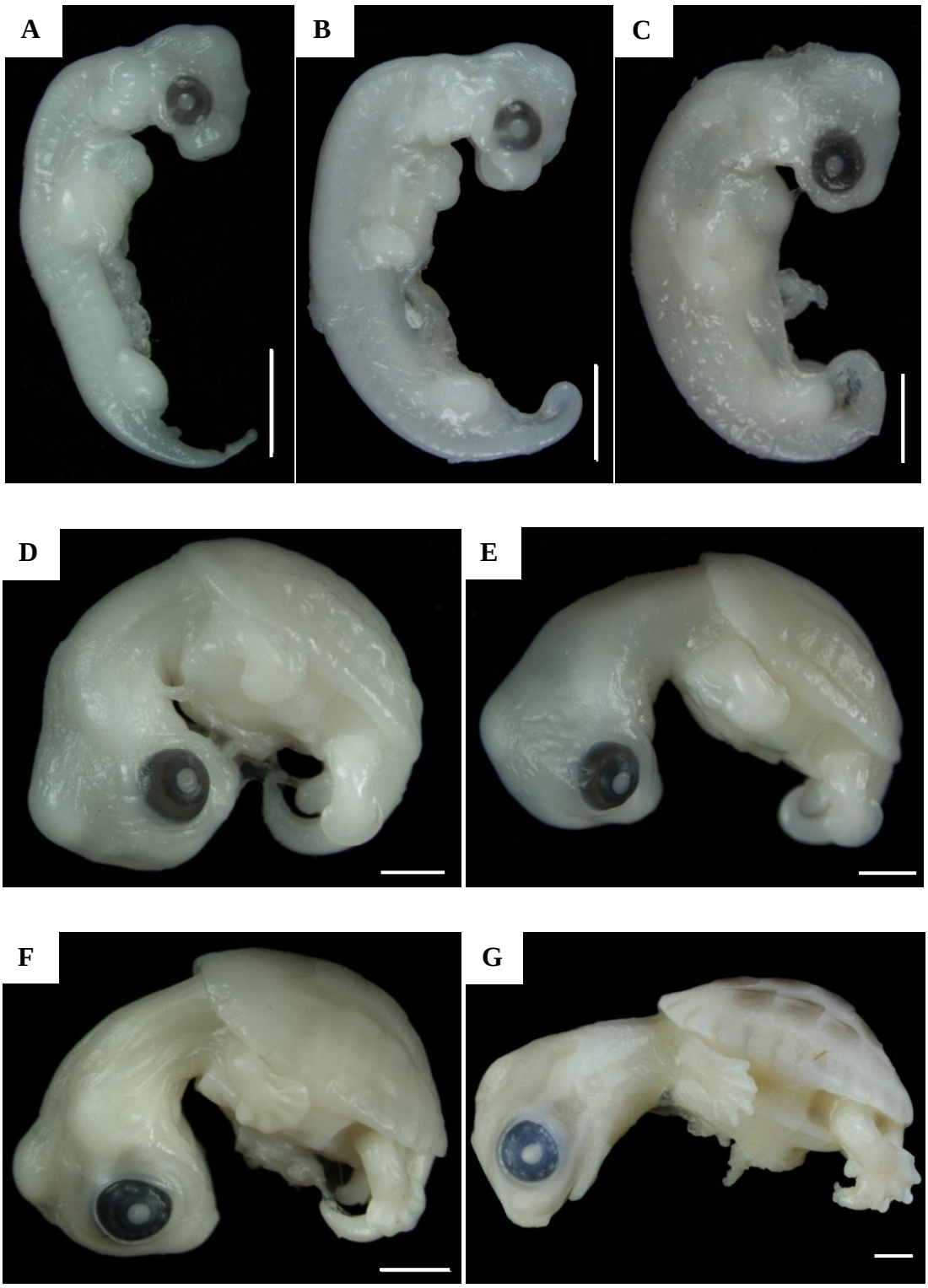


Figure 4.4: Photographs of embryonic stages of the snail-eating turtle in lateral view A-G; (A) stage 12 (B) stage 13 (C) stage 14 (D) stage 15 (E) stage 16 (F) stage 17 (G) stage 18. Scale bar = 1 mm.

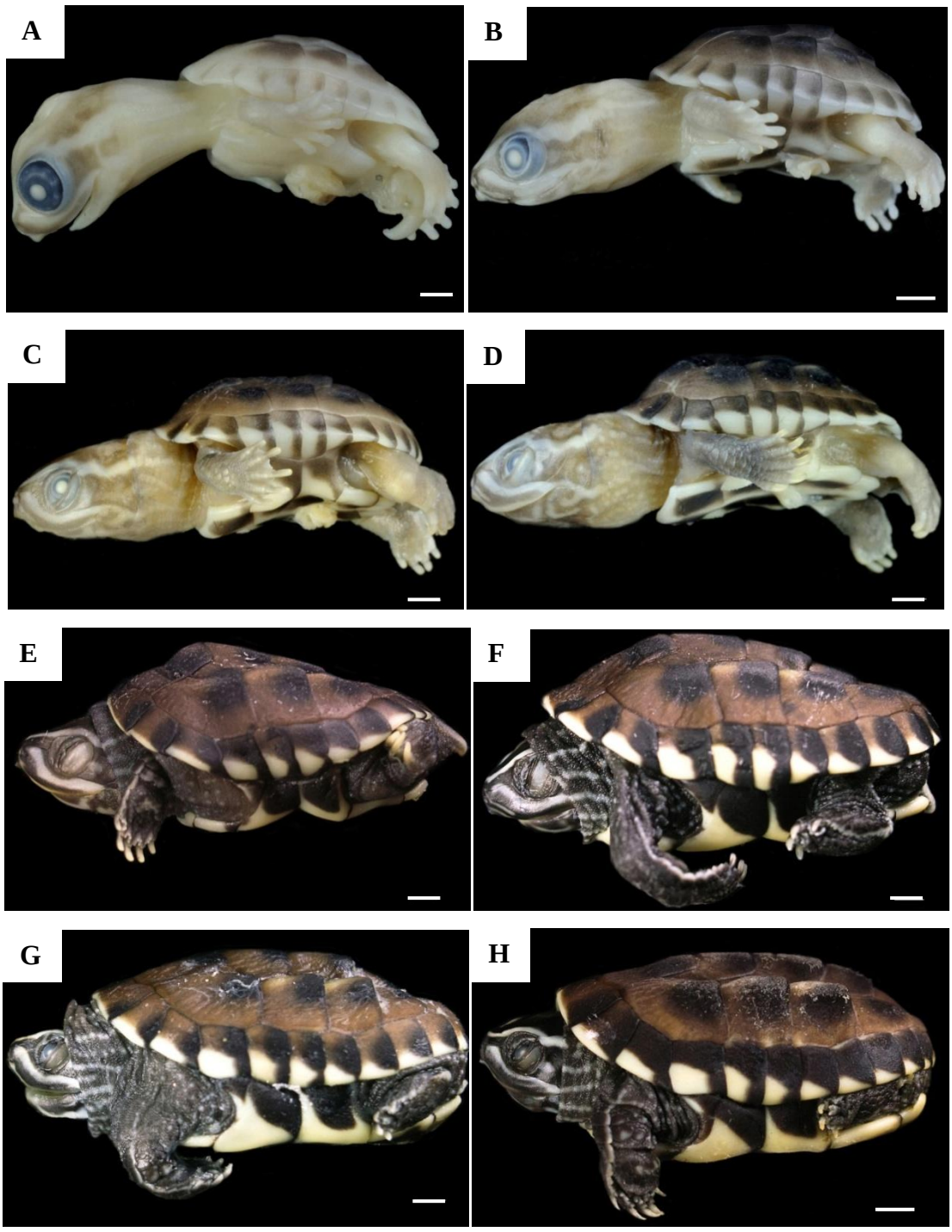


Figure 4.5: Photographs of embryonic stages of the snail-eating turtle in lateral view A-H; (A) stage 19 (B) stage 20 (C) stage 21 (D) stage 22 (E) stage 23 (F) stage 24 (G) stage 25 (H) stage 26. Scale bar = 1 mm.

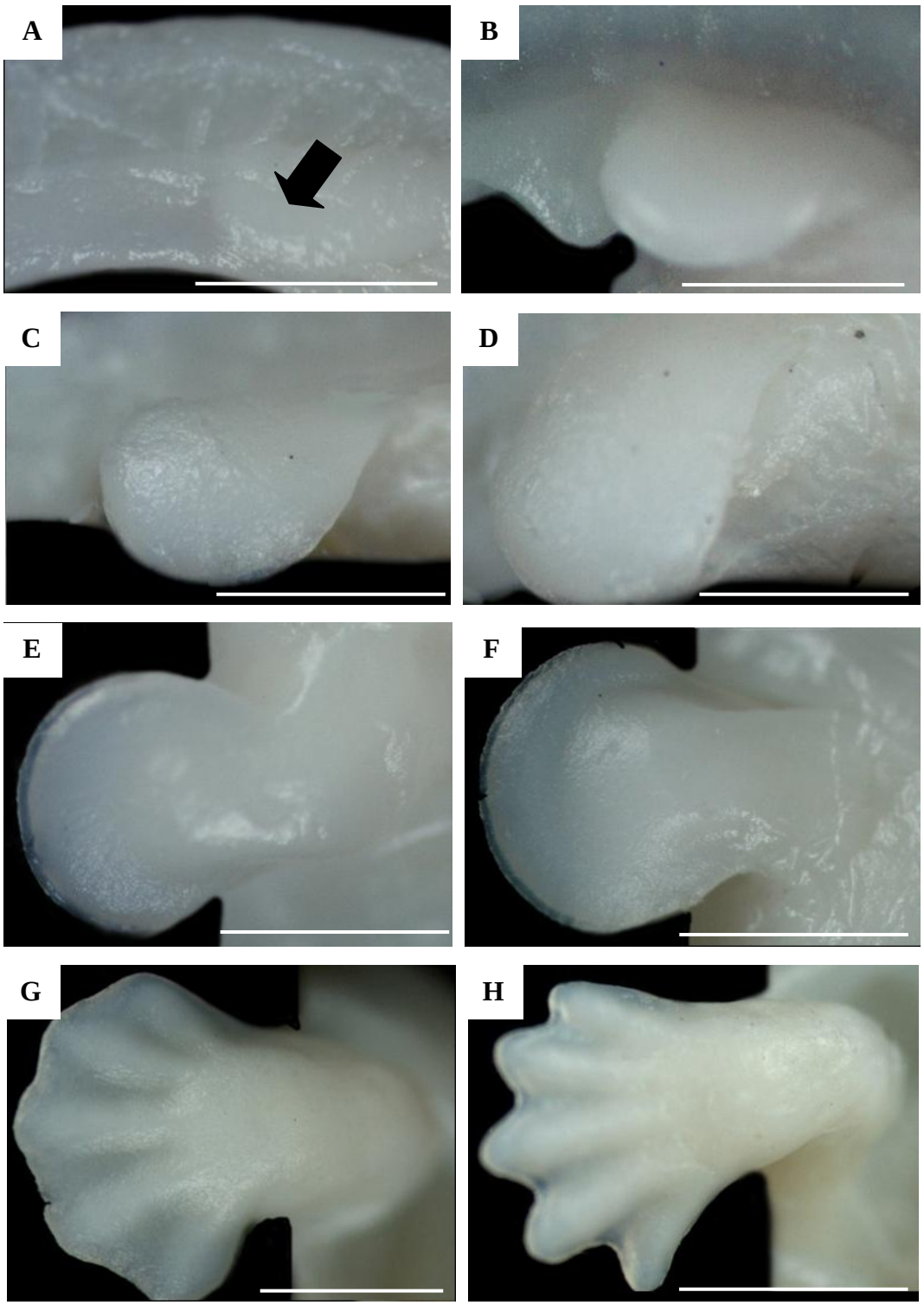


Figure 4.6: Photographs of forelimb in embryonic stages of the snail-eating turtle (A) stage 11, black arrow = forelimb (B) stage 12 (C) stage 13 (D) stage 14 (E) stage 15 (F) stage 16 (G) stage 17(H) stage 18. Scale bar = 1mm.

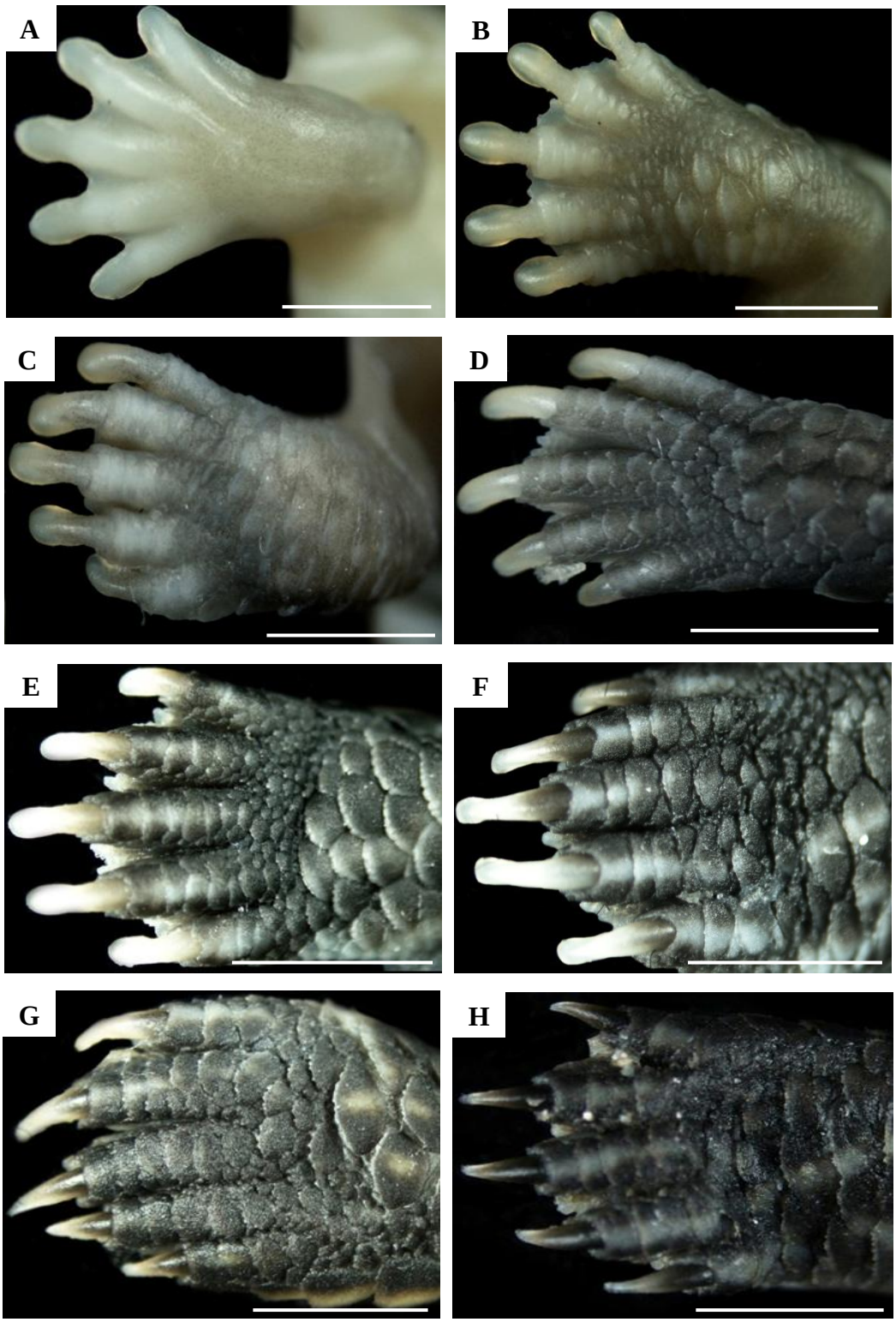


Figure 4.7: Photographs of forelimb in embryonic stages of the snail-eating turtle (A) stage 19 (B) stage 20 (C) stage 21 (D) stage 22 (E) stage 23 (F) stage 24 (G) stage 25 (H) stage 26. Scale bar = 1 mm.

4.3.2 Change in body size of *M. macrocephala* embryo

A total of 182 embryos were obtained from 237 eggs incubated at 29 °C. Average size (crown-rump length and carapace length) of *M. macrocephala* are shown in Table 4.1.

Table 4.1: Morphometry of body size of *M. macrocephala* embryos (stages 6-26)

Stage	Incubation periods (days)	Crown-rump length (mm)	Carapace length (mm)
6	15	3.57 (n=1)	–
7	18±5	4.23±0.29 (n=2)	–
8	19	5.31 (n=1)	–
9	29±6	5.34±0.06 (n=2)	–
10	29	5.41 (n=1)	–
11	29	5.09 (n=1)	–
12	29	5.63 (n=1)	–
13	29±10	6.72±0.12 (n=2)	–
14	29±6	7.90±0.32 (n=4)	–
15	33±5	–	5.89±0.85 (n=2)
16	36	–	6.18 (n=1)
17	36±6	–	7.59±0.75 (n=4)
18	44±3	–	9.24±0.62 (n=5)
19	50±6	–	11.67±1.03 (n=4)
20	53±4	–	14.52±0.72 (n=4)
21	57±10	–	17.16±1.02 (n=5)
22	67±13	–	20.03±1.02 (n=4)
23	61±9	–	24.24±1.50 (n=12)
24	83±12	–	29.59±2.76 (n=18)
25	129±25	–	31.22±2.18 (n=82)
26	115±20	–	31.34.±2.17 (n=26)

4.4 Conclusion

A series of developmental stages of *M. macrocephala*, a native and the most common freshwater species in Thailand, has been established with 21 discrete embryonic stages (stages 6 through 26) based on morphological examinations of 182 embryos from 237 eggs incubated at 29.0±1.3 °C, and referenced to the widely used stages of turtle development (Yntema, 1968; Tokita and Kuratani, 2001). Using this developmental stages as parameters of growth, it was found that the developmental stage of *M. macrocephala* embryos was comparable with the reference species.

CHAPTER V

EFFECT OF INCUBATION TEMPERATURE ON SOMATIC DEVELOPMENT OF THE SNAIL-EATING TURTLE *MALAYEMYS MACROCEPHALA*

5.1 Introduction

The Intergovernmental Panel on Climate Change (IPCC, 2007) reported that global warming, a gradual increase in average temperature on the earth, can change both physical and biological environments and affect survival of organisms. Organisms that cannot adjust oneself to suit with the changed environment may die down and become extinct (Walther et al., 2002). In reptile, temperature is an important ecological factor (Zug et al., 2001), and an incubation temperature has been demonstrated to have profound effects on embryonic development (Deeming and Ferguson, 1991). Incubation at high temperature could accelerate embryonic development compared to incubation at lower temperatures (Pina and Larriera, 2002; Georges et al., 2005).

In freshwater turtle, incubating eggs at different temperature could affect the incubation period and pattern of development (Pieau and Dorizzi, 1981; Packard et al., 1987). Many reports showed that increased incubation temperature resulted in earlier hatching (Ewert, 1979; 1985). The incubation temperature is also an important variable affecting hatching rate and development of freshwater turtle (Deeming and Ferguson, 1991; Grant et al., 2003) and can determine sex ratio of a turtle population (Bull et al., 1990; Willingham, 2005). As a result, an ecological model predicted that climate change may exhibit long-term impact on freshwater turtle populations (Parrott and Logan, 2010).

In addition, population dynamics of the freshwater could be affected by deformities of the turtle (Davy and Murphy, 2009). Although developmental abnormalities with varying severity are not uncommon in wild populations of freshwater turtles (Ewert, 1979; MacCulloch, 1981; Pavaliko, 1986; Bell et al., 2006), thermal conditions during incubation were shown to have significant effects on embryonic growth and development in turtles (Deeming and Ferguson, 1991). Incubation at temperatures below or above optimal thermal range for significant periods of time could result in embryonic malformation (Booth, 2006).

In Thailand, although several species of freshwater turtles are present (Nutphand, 1979; Thirakhupt and van Dijk, 1994), an extent of their susceptibility to temperature change is unknown due to the lack of information on their development patterns. The snail-eating turtle, *Malayemys macrocephala*, is one of the native species and the most common freshwater turtle in Thailand (Brophy, 2004). As a result, sufficient baseline information about this species is available in regard to the natural history (Nutphand, 1979), population biology (Srinarumol, 1995) and ecotoxicology (Keithmaleesatti, 2008). Current research thus aimed to examine effect of incubation temperature on somatic development of *M. macrocephala* in order to provide prediction on the potential effects of increased regional temperature on this native freshwater turtle species.

5.2 Materials and Methods

5.2.1 Turtle egg collection

Visual encounter surveys for nests of *M. macrocephala* were carried out in rice fields (UTM Zone 47P: 0653086-0659077 and 1583552-1591014) at Bang Ban District, Phra Nakhon Si Ayutthaya Province, an important breeding ground of this turtle species in central part of Thailand (Keithmaleesatti, 2008), during December 2011 to February 2012. Based on 5 field surveys, a total of 712 eggs from 126 clutches were collected. Eggs were individually numbered, placed in a styrofoam box and transported to a laboratory at the Department of Biology, Faculty of Science, Chulalongkorn University. All portions of this research project involving turtle egg collection and animal subjects had been approved by the Chulalongkorn University Animal Care and Use Committee (Protocol Review Number 1323005).

5.2.2 Turtle egg incubation

In the laboratory, eggs were cleaned and weighed on an electronic balance (Tanita, accuracy 0.01 g). A total of 712 eggs were randomly placed in plastic boxes containing moisted vermiculite (1 part vermiculite : 1 part distilled water; weight : volume). Eggs were kept in microprocessor-controlled incubators (Siam Incubators System, Bangkok, Thailand) at three temperatures including 26°C (n=237), 29°C (n=237) and 32°C (n=238). The eggs were buried completely in the medium and the boxes were covered with plastic lids. A tray of water was placed inside each incubator to controlled relative humidity in excess of 80%. Temperature and relative humidity inside the box were monitored using a data logger (HAXO-8, LogTag Recorders, Auckland, New Zealand). Eggs were randomly sampled, weighed and dissected on weekly basis until hatch.

5.2.3 Change in weight of turtle egg

Weight of turtle egg tended to be reduced after incubation, possibly due to water loss (Tracy et al., 1978). Percentage of weight loss was determined based on weight of eggs before incubation and weight of eggs after incubation at different temperature (26°C, 29°C and 32°C). The percentage of egg weight loss was calculated as follows:

$$\text{Weight loss (\%)} = \frac{\text{egg weight before incubation} - \text{egg weight after incubation}}{\text{egg weight before incubation}}$$

5.2.4 Relative weight of turtle hatchling

To monitor effect of incubating temperature on hatching weight, relative weight of hatchling from eggs incubated at the different temperature (26°C, 29°C and 32°C) were calculated from a formula below:

$$\text{Relative weight} = \frac{\text{hatching weight (g)}}{\text{egg weight (g)}}$$

5.2.5 Growth and development of turtle embryo

After dissection, developmental stage of the snail-eating turtle (see Chapter IV) was studied in reference to the widely use developmental stages of *Chelydra serpentina* (Yntema, 1968) and *Pelodiscus sinensis* (Tokita and Kuratani, 2001). Body length was measured by a vernier caliper (Mitutoyo, accuracy 0.01 mm) and used as an indicative of size of embryo. Crown-rump length (CRL) was measured at stage 3 to 14 and carapace length (CL) was measured at stage 15 to hatching stage 26 (Figures 5.1). After measurement, embryos were subjected to euthanasia by injection with pentobarbital sodium at a dose of 600 mg/kg

intraperitoneally. Embryo were fixed in Davidson's fixative for 24 hours and then preserved in 70% ethanol.

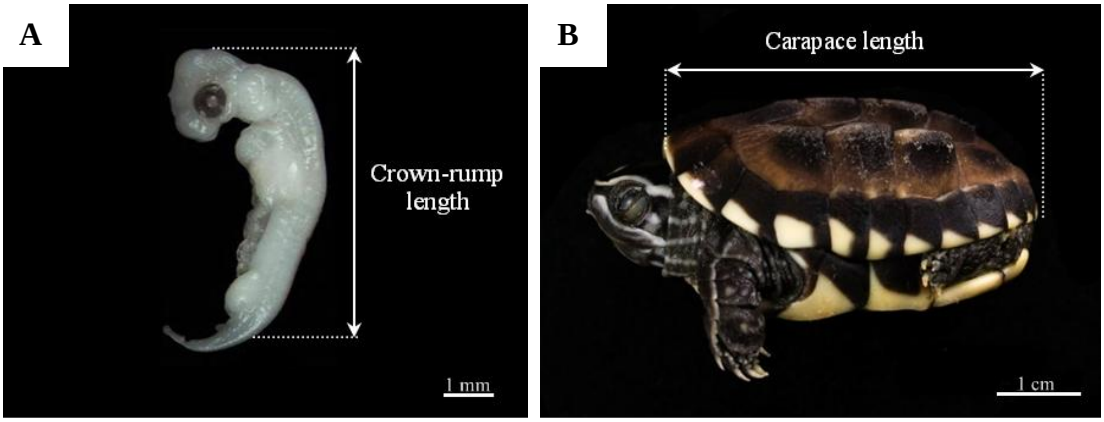


Figure 5.1: Measurement for body length as an indicative of size (A) crown-rump length (CRL) for embryonic stage 3 to 14 and (B) carapace length (CL) for embryonic stage 15 to 26.

5.2.6 Developmental abnormality of turtle embryo

Morphological examination showed that developmental abnormalities occurred with turtle embryos incubated at these three temperatures. The abnormalities were classified into three categories in reference to the widely use type of deformities in embryos of *Chelydra serpentina* and *Chrysemys picta* (Bell et al., 2006) as follows. First, minor deformities that are not likely to affect survival included narrow body (Figure 5.2A). Second, moderate deformities that can lower the chance of survival included 1) scoliosis 2) missing eye 3) flat skull and misshapen carapace, and 4) deformed body (Figure 5.2B-E). Third, lethal deformities that greatly reduce the chance of survival included dwarf (Figure 5.2F).

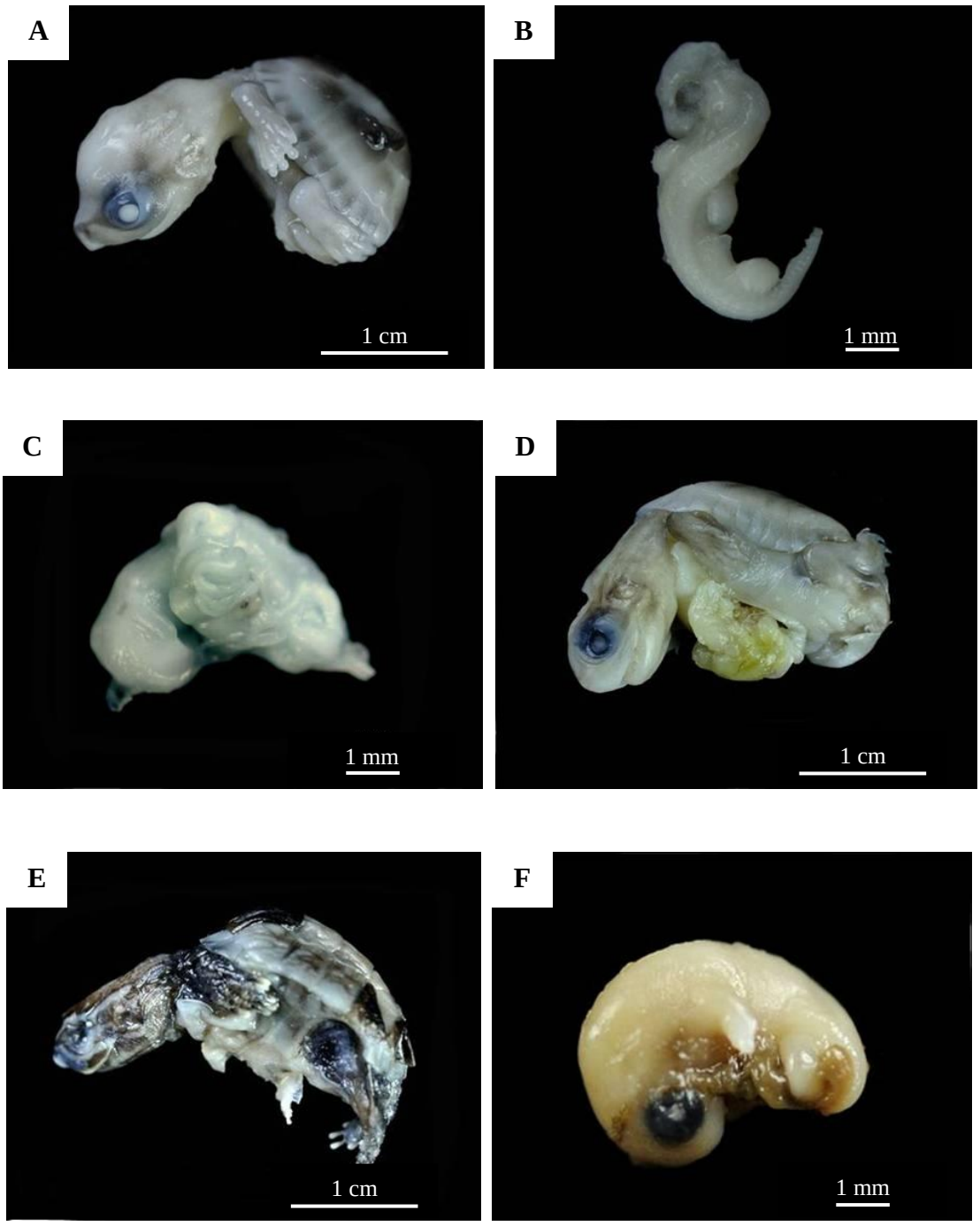


Figure 5.2: Types of deformities found in *M. macrocephala* embryos (A) narrow body (B) scoliosis (C) missing eye (D) flat skull and misshapen carapace (E) deformed body (F) dwarf.

5.2.7 Statistical analyses

Mean incubation period and mean relative weight of hatchling were compared among incubating temperature using one-way ANOVA followed by Bonferroni test.

Changes in egg weight were compared among temperature by analysis of covariance (ANCOVA) using time of incubation as a covariable, followed by Bonferroni test.

Relationships between incubation time vs. crown-rump length, carapace length and embryonic stage were analyzed by logistic regression analysis. Afterward, slopes of the trend lines were compared among temperature by analysis of variance followed by Tukey multiple comparisons.

Eggs/embryos from each incubating temperature were classified into three categories including 1) non fertile egg 2) normal embryo and 3) deformed embryo. A 3x3 contingency tables was used to determine association between temperature (26, 29 and 32 °C) and normality in development (non fertile egg, normal embryo and deformed embryo).

Statistical analyses undertaken in this study were carried out with SPSS version 11.5, except slope comparison and contingency table analysis that were manually calculated according to Zar (1998). Statistically significant difference is reported at $p<0.05$.

5.3 Results and Discussion

5.3.1 Effect of temperature on incubation period

Table 5.1 shows that the incubation period, or time required for egg to hatch, of *M. macrocephala* eggs incubated at three different temperatures ranged from 78-150 days. The result indicated that there was no significantly different among incubation periods at different incubation temperature (one way ANOVA, $p>0.05$). Mean ± S.E.M. of incubation periods at 26, 29 and 32 °C were 115±11.3, 115±20.3 and 109±17.8 days, respectively.

The incubation period of *M. macrocephala* egg was relative longer than other freshwater turtles found in tropical region such as *Trachemys scripta* (65-75 days; Ernst and Barbour, 1989) and *Batagur baska* (70-112 days; Ernst and Barbour, 1989). However, potential effect of incubating temperature on incubation period was not found in this study.

Table 5.1: Incubation periods or time requires for *M. macrocephala* to hatch (mean±S.E.M.) at different incubation temperatures

Temperature	Range of incubation period (days)	Average incubation period (days)
Low temperature (26 °C)	89-134 (n=40)	115±11.3
Pivotal temperature (29 °C)	78-150 (n=26)	115±20.3
High temperature (32 °C)	85-131 (n=9)	109±17.8

5.3.2 Effect of incubation temperature on egg weight loss

Change in percentage of egg weight loss during 24 weeks of incubation is presented in Figure 5.3 and Table 5.2. The change in egg weight was not significantly different among incubating temperatures group (ANCOVA, $p>0.05$). Since egg weight loss during incubation was mainly due to water loss (Tracy et al., 1978), the result suggested that water loss was not significantly different among incubating temperatures. As a result, effect of humidity on embryonic development was unlikely (Packard, 1991).

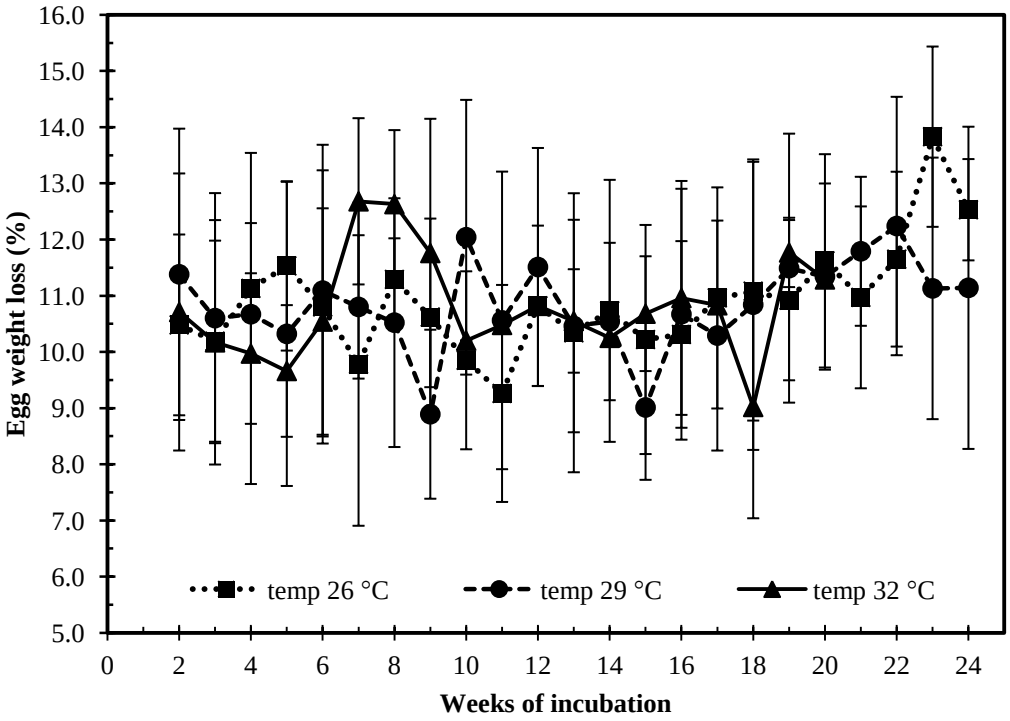


Figure 5.3: Egg weight loss during incubation (Mean±S.E.M.) of *M. macrocephala* eggs incubated at different temperatures (26°C, 29°C and 32°C) for 24 weeks. There was no significant difference among temperatures (ANCOVA, $p>0.05$).

Table 5.2: Egg weight loss during incubation (Mean±S.E.M.) of *M. macrocephala* eggs incubated at different temperatures (26°C, 29°C and 32°C) for 24 weeks

Incubation time	**Percentage of egg weight loss (mean±S.E.M.)**		
	Temperature		
	26 °C	29 °C	32 °C
2nd week	10.48±1.61 (n=5)	11.38±2.59 (n=5)	10.71±2.47 (n=6)
3rd week	10.19±1.79 (n=5)	10.60±2.23 (n=5)	10.17±2.18 (n=8)
4th week	11.13±2.41 (n=6)	10.67±0.73 (n=5)	9.97±2.32 (n=5)
5th week	11.53±1.51 (n=7)	10.32±2.71 (n=7)	9.66±1.17 (n=6)
6th week	10.80±2.43 (n=6)	11.09±2.60 (n=7)	10.54±2.02 (n=5)
7th week	9.78±2.88 (n=7)	10.80±1.28 (n=8)	12.68±1.48 (n=4)
8th week	11.29±0.73 (n=7)	10.52±2.21 (n=8)	12.63±1.42 (n=3)
9th week	10.62±1.75 (n=6)	8.89±1.50 (n=8)	11.76±2.39 (n=4)
10th week	9.85±1.58 (n=6)	12.04±2.44 (n=8)	10.20 (n=1)
11th week	9.26±1.93 (n=6)	10.56±2.65 (n=6)	10.48 (n=1)
12th week	10.82±1.43 (n=8)	11.51±2.12 (n=11)	10.82±0.92 (n=3)
13th week	10.34±2.48 (n=11)	10.46±1.89 (n=11)	10.55±2.46 (n=4)
14th week	10.73±2.33 (n=11)	10.54±1.40 (n=9)	10.25 (n=1)
15th week	10.22±2.04 (n=21)	9.01±1.29 (n=9)	10.68±1.02 (n=2)
16th week	10.31±1.66 (n=18)	10.67±2.23 (n=9)	10.96±2.08 (n=4)
17th week	10.96±1.97 (n=10)	10.29±2.05 (n=14)	10.83 (n=1)
18th week	11.08±2.30 (n=21)	10.842.59 (n=13)	9.02±1.98 (n=4)
19th week	10.92±1.43 (n=7)	11.492.39 (n=11)	11.77±0.62 (n=2)
20th week	11.62±1.90 (n=6)	11.34±1.66 (n=7)	–
21st week	10.97±1.62 (n=5)	11.79±1.33 (n=7)	–
22nd week	11.65±1.56 (n=6)	12.24±2.30 (n=7)	–
23rd week	13.83±1.60 (n=4)	11.13±2.33 (n=8)	–
24th week	12.53±0.90 (n=3)	11.14±2.87 (n=3)	11.29 (n=1)

5.3.3 Effect of incubation temperature on relative weight

At hatching, weight of each turtle was calculated in relation to egg weight. It was found that relative weight of hatchlings was not significantly different among temperatures (Figure 5.4 and Table 5.3). The result suggested that the incubation temperatures used in this study seems to similarly influence the rate of embryonic development (Deeming and Ferguson, 1991).

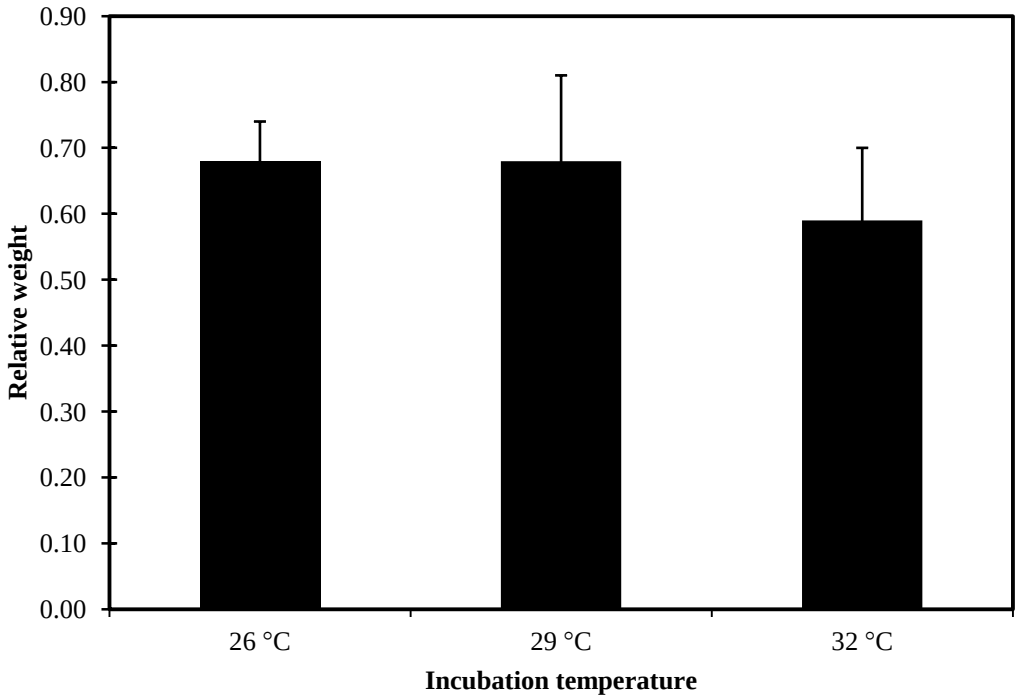


Figure 5.4: Relative weight of hatchling from eggs incubated at different temperatures (26°C, 29°C, and 32°C). There was no significant difference among incubating temperature (one-way ANOVA, $p>0.05$).

Table 5.3: Relative weight for *M. macrocephala* hatchling (mean±S.E.M.) after incubation at three different incubation temperatures

Temperature	Average egg weight (g)	Average hatchling weight (g)	Average relative weight
26 °C (n=40)	11.37±1.88	7.75±1.54	0.68±0.06
29 °C (n=26)	10.92±1.97	6.65±1.30	0.68±0.13
32 °C (n=9)	11.20±0.91	6.56±0.73	0.59±0.11

5.3.4 Effect of temperature on growth and development

Growth of *M. macrocephala* embryos was assessed from crown-rump length for embryonic stage 3-14 and carapace length for embryonic stage 15-26 during these 24 weeks of incubation. To monitor for early growth, change in crown-rump length was examined during the 2nd week to the 8th week of incubation (Table 5.4). According to the logistic regression analyses, significant relationships between incubation time and crown-rump length of *M. macrocephala* (Figure 5.5) were found in turtle incubated at 26 and 32 °C with R^2 values of 0.68 and 0.87, respectively (Table 5.7). However, there was no significant relationship between incubation time and crown-rump length of *M. macrocephala* embryos incubated at 29 °C. As a result, data on crown-rump length was excluded from further slope comparison.

To monitor for growth until hatch, change in carapace length was examined during the 3rd week to the 24th week of incubation (Table 5.5). In logistic regression analyses, significant relationship between incubation time and carapace length of *M. macrocephala* (Figure 5.6) were found in these three incubating temperatures with R^2 values of 0.91 (26 and 32 °C) and 0.87 (29 °C; Table 5.7).

Development of *M. macrocephala* embryo was monitored from an advancing in embryonic stage during the 2nd week to the 24th week of incubation (Table 5.6). Significant logistic regressions between incubation time and developmental stage of *M. macrocephala* (Figure 5.7) were found in these three incubating temperatures (26, 29 and 32 °C) with R^2 values of 0.95, 0.89 and 0.85, respectively (Table 5.7).

Based on logistic regression lines between incubation time vs. carapace length (Figure 5.6) and incubation time vs. developmental stage (Figure 5.7), slopes of these trend lines were compared among incubating temperatures by ANOVA followed by

Tukey multiple comparisons. It was found that significant difference in slopes was found in every pair-wise comparison ($p<0.05$, Table 5.8), indicating influence of temperature on somatic development of the snail-eating turtle.

Similar to previous study (Vinegar, 1973), it was found that development (based on embryonic stage) and growth (based on carapace length) of *M. macrocephala* embryos showed significant logistic relationship with incubation time. For reptilian eggs, incubation at different temperatures generally results in temperature-induced differences in growth pattern and hatching morphology (Hutton, 1987; Packard et al., 1988). In this study, logistic regression and slope comparison also showed that embryos derived from different incubating temperature had significantly different growth patterns from each other. Incubation at high temperature (32 °C) resulted in relatively faster growth and development of embryos compared to other temperature (26 °C and 29°C). These results confirmed previous observations that temperature is an important environment factor that affects developmental rate of turtle embryo (Valenzuela, 2001; Zhu et al., 2006).

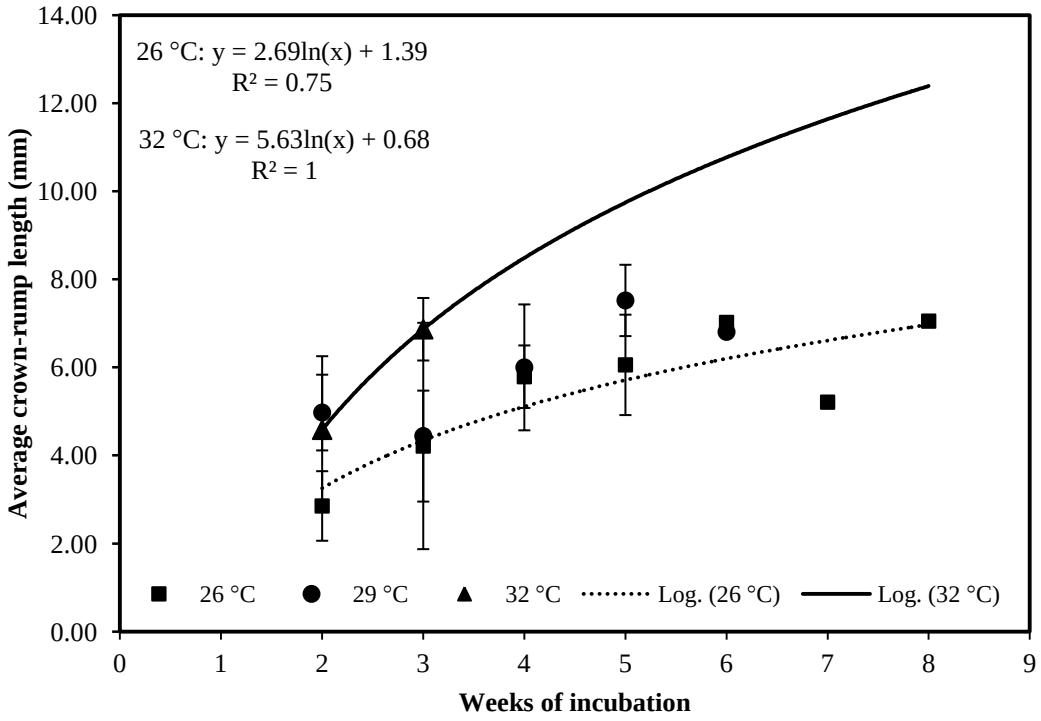


Figure 5.5: Effect of incubation temperature on growth pattern (based on crown-rump length) of *M. macrocephala* embryos. Relationship between incubation periods and crown-rump length were analyzed by logistic regression analysis. Significant relationships ($p<0.05$) were found only in turtle incubated at 26 and 32 °C.

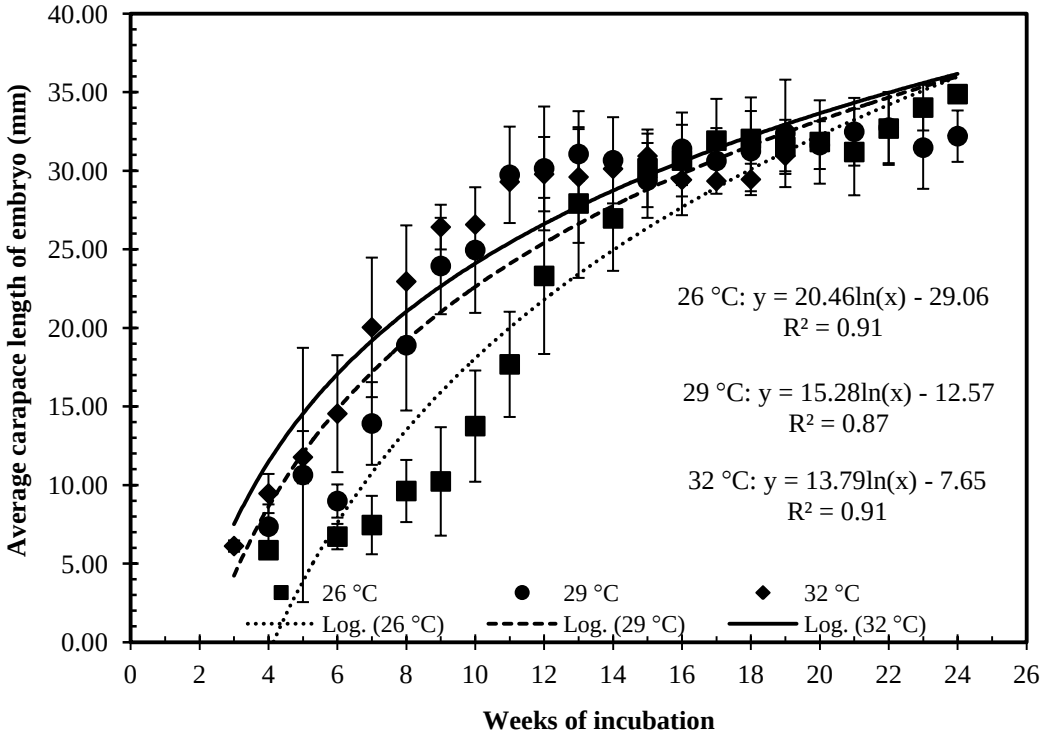


Figure 5.6: Effect of incubation temperature on growth pattern (based on carapace length) of *M. macrocephala* embryos. Significant relationships between incubation time and carapace length (logistic regression analysis, $p<0.05$) were found in every incubating temperatures.

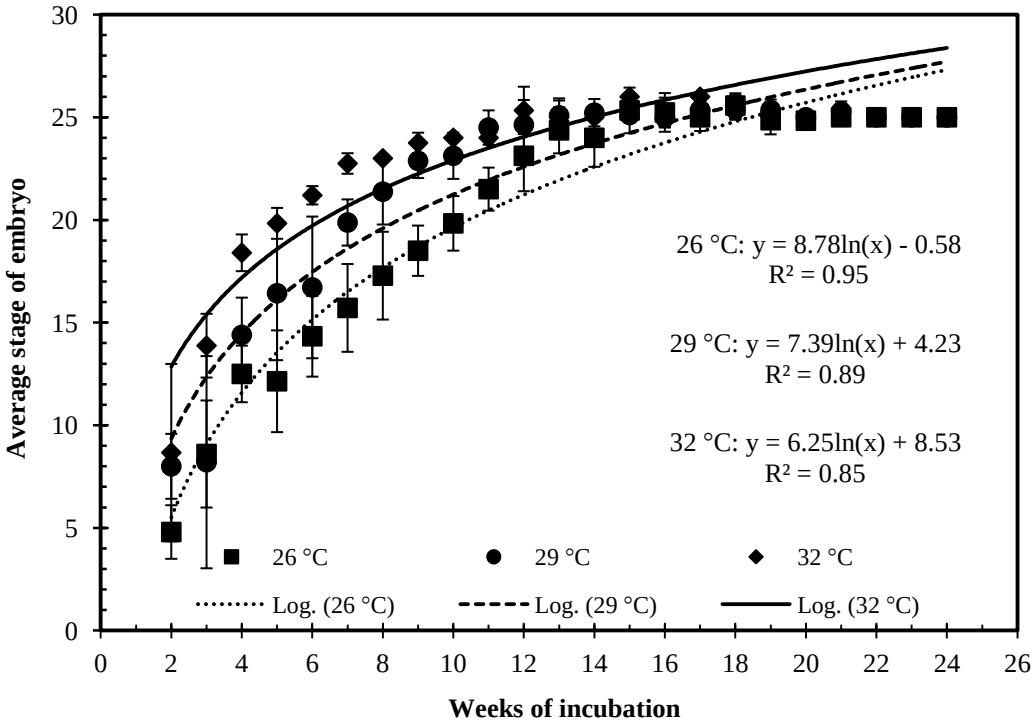


Figure 5.7: Effect of incubation temperature on growth pattern (based on embryonic stage) of *M. macrocephala* embryos. Significant relationships between incubation time and embryonic stage (logistic regression analysis, $p<0.05$) were found in every incubating temperatures.

Table 5.4: Change in crown-rump length (mean±S.E.M.) of *M. macrocephala* embryos (stages 3-14) after incubation at different temperatures for 8 weeks

Incubation time	**Crown-rump length (mm) (mean±S.E.M.)**		
	Temperature		
	26 °C	29 °C	32 °C
2nd week	2.88±0.79 (n=7)	4.72±0.86 (n=5)	4.52±1.67 (n=6)
3rd week	4.81±1.26 (n=5)	4.52±2.57 (n=6)	7.10±0.71 (n=5)
4th week	5.67±0.71 (n=5)	7.27±1.43 (n=3)	–
5th week	6.33±1.14 (n=6)	7.39±0.81 (n=2)	–
6th week	5.30±0.30 (n=2)	5.46 (n=1)	–
7th week	5.21 (n=1)	–	–
8th week	7.05 (n=1)	–	–

Table 5.5: Change in carapace length (mean±S.E.M.) of *M. macrocephala* embryos (stages 15-26) after incubation at different temperatures for 24 weeks

Incubation time	Carapace lengths (mm) (mean±S.E.M.)		
	Temperature		
	26 °C	29 °C	32 °C
3rd week	–	–	6.11±0.36 (n=3)
4th week	5.85±0.00 (n=1)	7.35±1.41 (n=2)	9.46±1.24 (n=5)
5th week	–	10.64±10.31 (n=5)	11.77±1.66 (n=6)
6th week	6.72±0.81 (n=4)	8.98±1.06 (n=6)	14.54±3.72 (n=5)
7th week	7.45±1.86 (n=6)	13.91±2.63 (n=8)	20.03±4.44 (n=4)
8th week	9.62±1.98 (n=6)	18.89±4.15 (n=8)	22.95±3.57 (n=3)
9th week	10.23±3.45 (n=6)	23.94±3.07 (n=8)	26.41±1.42 (n=4)
10th week	13.75±3.54 (n=6)	24.95±4.00 (n=8)	26.57 (n=1)
11th week	17.68±3.35 (n=6)	29.74±3.07 (n=6)	29.29 (n=1)
12th week	23.30±4.97 (n=8)	30.14±3.94 (n=11)	29.78±2.37 (n=3)
13th week	27.91±4.73 (n=11)	31.06±1.71 (n=11)	29.60±4.19 (n=4)
14th week	26.96±3.34 (n=11)	30.66±2.74 (n=9)	30.12 (n=1)
15th week	30.15±2.47 (n=21)	29.38±2.38 (n=9)	30.93±1.43 (n=2)
16th week	30.64±2.28 (n=18)	31.39±2.31 (n=9)	29.42±2.25 (n=4)
17th week	31.91±2.66 (n=10)	30.62±2.09 (n=14)	29.34 (n=1)
18th week	32.02±2.64 (n=21)	31.25±2.55 (n=13)	29.45±1.00 (n=4)
19th week	31.52±1.72 (n=7)	32.37±3.42 (n=11)	30.95±0.98 (n=2)
20th week	31.83±2.65 (n=6)	31.63±1.52 (n=7)	–
21st week	31.19±2.75 (n=5)	32.48±2.16 (n=7)	–
22nd week	32.68±2.31 (n=6)	32.74±2.26 (n=7)	–
23rd week	34.02±1.46 (n=4)	31.47±2.62 (n=8)	–
24th week	34.87±0.53 (n=3)	32.20±1.64 (n=3)	32.23 (n=1)

Table 5.6: Advancing in embryonic stage (mean±S.E.M.) of *M. macrocephala* embryo after incubation at different temperatures for 24 weeks

Incubation time	Embryonic stage (mean±S.E.M.)		
	Temperature		
	26 °C	29 °C	32 °C
2nd week	5±1.30 (n=5)	8±1.58 (n=5)	9±4.32 (n=6)
3rd week	9±2.61 (n=5)	10±5.17 (n=5)	14±1.55 (n=8)
4th week	13±1.38 (n=6)	14±1.82 (n=5)	18±0.89 (n=5)
5th week	12±2.48 (n=7)	16±3.26 (n=7)	20±0.75 (n=6)
6th week	14±1.97 (n=6)	17±3.45 (n=7)	21±0.45 (n=5)
7th week	16±2.14 (n=7)	20±1.13 (n=8)	23±0.50 (n=4)
8th week	17±2.14 (n=7)	21±1.60 (n=8)	23±0.00 (n=3)
9th week	19±1.22 (n=6)	23±0.83 (n=8)	24±0.50 (n=4)
10th week	20±1.33 (n=6)	23±1.13 (n=8)	24 (n=1)
11th week	22±1.05 (n=6)	25±0.84 (n=6)	24 (n=1)
12th week	23±1.73 (n=8)	25±1.21 (n=11)	25±1.15 (n=3)
13th week	24±1.12 (n=11)	25±0.83 (n=11)	25±0.82 (n=4)
14th week	24±1.41 (n=11)	25±0.67 (n=9)	25 (n=1)
15th week	25±1.11 (n=21)	25±0.33 (n=9)	26±0.00 (n=2)
16th week	25±0.94 (n=18)	25±0.50 (n=9)	25±0.71 (n=4)
17th week	25±0.67 (n=10)	25±0.50 (n=14)	26 (n=1)
18th week	26±0.60 (n=21)	25±0.51 (n=13)	26±0.58 (n=4)
19th week	25±0.69 (n=7)	25±0.50 (n=11)	25±0.00 (n=2)
20th week	25±0.41 (n=6)	25±0.00 (n=7)	–
21st week	25±0.00 (n=5)	25±0.49 (n=7)	–
22nd week	25±0.00 (n=6)	25±0.00 (n=7)	–
23rd week	25±0.00 (n=4)	25±0.00 (n=8)	–
24th week	25±0.00 (n=3)	25±0.00 (n=3)	25 (n=1)

Table 5.7: Logistic models for regression lines of incubation time and growth pattern of *M. macrocephala* based on crown-rump length, carapace length and developmental stage

Temperature	N	Slope of regression line	Y Intercept	R^2	p_{value}
Logistic regression between time and crown-rump length					
Low temperature (26 °C)	26	2.19	1.39	0.68	0.001
Pivotal temperature (29 °C)	17	1.79	3.51	0.32	0.241
High temperature (32 °C)	11	6.57	0.10	1.00	0.011
Logistic regression between time and carapace length					
Low temperature (26 °C)	166	20.46	-29.06	0.91	0.000
Pivotal temperature (29 °C)	169	15.28	-12.57	0.87	0.000
High temperature (32 °C)	54	13.79	-7.65	0.91	0.000
Logistic regression between time and embryonic stage					
Low temperature (26 °C)	192	8.78	-0.58	0.95	0.000
Pivotal temperature (29 °C)	186	7.39	4.23	0.89	0.000
High temperature (32 °C)	65	6.25	8.53	0.85	0.000

Table 5.8: Comparisons for temperature-related difference in slopes (ANOVA and Tukey multiple comparisons) of logistic regression lines between incubation time and development of *M. macrocephala* based on carapace length and embryonic stage

Slope comparison	N	F_{cal}	p_{value}	q	p_{value}
Comparison on slope of logistic regression between incubation time and carapace length					
26°C vs. 29°C	335	4.02	p<0.05	5.79	p<0.05
26°C vs. 32°C	220	4.02	p<0.05	2.62	p<0.05
29°C vs. 32°C	223	4.02	p<0.05	3.57	p<0.05
Comparison on slope of logistic regression between incubation time and embryonic stage					
26°C vs. 29°C	378	43.21	p<0.05	8.11	p<0.05
26°C vs. 32°C	257	43.21	p<0.05	27.25	p<0.05
29°C vs. 32°C	251	43.21	p<0.05	9.76	p<0.05

5.3.5 Effect of temperature on developmental abnormality

Upon dissection, eggs and embryos from each incubating temperature were classified into three categories including 1) non fertile egg 2) normal embryo and 3) deformed embryo (Table 5.9). The results showed that proportions of non fertile eggs at these temperatures were relatively similar (7.59% at 26 °C, 4.27% at 29 °C and 10.50% at 32 °C). However, incubation at 26 °C and 29 °C resulted in higher proportion of normal embryos (81.01% and 78.48%, respectively), while incubation at 32 °C resulted in much lower proportion of normal embryo (27.31%). As a result, proportions of deformed embryo at these temperatures were markedly different (0.84% at 26 °C, 2.53% at 29 °C and 30.25% at 32 °C). The 3 x 3 contingency table analysis (Table 5.10) showed a significant association between incubating temperature and the normality of the embryos ($\chi^2 = 202.38$, d.f. = 4, $p<0.05$).

It is of interest to note that incubation at high temperature (32 °C) seemed to be harmful to turtle embryos since higher proportion of deformities was evidenced. Obviously, reptilian eggs cannot be incubated at extremely high or low temperatures for extended period of time since it could increase embryonic deformities (Du and Ji, 2003; Sinervo and Adolph, 1989). In turtle, thermal condition during incubation is known to influence incubation time, metabolism, sex, embryo mortality, hatching success and size at hatching (Standing et al., 2000). The results from this study provide further evidence that increase in incubating temperature could result in higher incidence of developmental abnormality.

Table 5.9: Incidences of developmental abnormality of *M. macrocephala* embryos incubated at three different temperatures.

Stage of eggs/embryos	Temperature			Total
	26 °C	29 °C	32 °C	
Total number of eggs	237	237	238	**712**
Non fertile	18	10	25	**53**
Normal embryos	192	186	65	**443**
Deformed embryo	2	6	72	**80**
Minor deformities				
Narrow body	1	1	2	
Moderate deformities				
Scoliosis	1	0	1	
Missing eye	0	1	4	
Skull flat, misshapen carapace	0	2	5	
Body deformed	0	2	55	
Lethal deformities				
Dwarf	0	0	5	

Table 5.10: A 3 x 3 contingency table analysis for association between incubating temperature and normality of eggs and embryos of *M. macrocephala*

Stage of eggs/embryos		Temperature			Total
		26 °C	29 °C	32 °C	
Non fertile	Observed (O)	18	10	25	53
	Expected (E)	19.51	18.58	14.91	
	$(0-E)^2/E$	0.11	3.96	6.83	
Normal embryos	Observed (O)	192	186	65	443
	Expected (E)	163.05	155.36	124.59	
	$(0-E)^2/E$	5.14	6.04	28.50	
Deformed embryo	Observed	2	6	72	80
	Expected	29.44	28.50	22.50	
	$(0-E)^2/E$	25.57	17.33	108.9	
$\chi^2 = 202.38$, d.f. = 4, $p<0.05$					

5.4 Conclusion

The incubation temperature is an important variable affecting development of freshwater turtle. In *M. macrocephala*, it is clear that incubating temperature could affect somatic development of turtle embryos. Incubation at high temperature (32 °C) resulted in significantly more rapid growth and development than other temperature (26 °C and 29°C). However, incubation at high temperature seemed to be harmful to embryo since higher incidence of deformities was found. Overall, the results suggest that increase in ambient temperature of turtle nest could affect somatic development of freshwater turtle.

CHAPTER VI

EFFECT OF INCUBATION TEMPERATURE ON GONADAL DEVELOPMENT OF THE SNAIL-EATING TURTLE *MALALEMYS MACROCEPHALA*

6.1 Introduction

Rise in global average temperature can change both physical and biological properties in environment and may affect survival of organisms (IPCC, 2007). Organisms that cannot adjust themselves to suit with the changed environment may die and become extinct (Walther et al., 2002). Freshwater turtles are susceptible species for temperature change since their embryonic development depends on incubation temperature. Therefore, the incubation temperature may affect hatching rate and development of freshwater turtle (Grant et al., 2003).

In addition, temperature is also regarded as an important factor in sex determination of many reptiles, including freshwater turtles, that exhibit temperature dependent sex determination or TSD (Valenzuela, 2004a). Different incubating temperature can induce significant difference in sex determination in turtle with TSD and can determine sex ratios of a turtle population (Bull et al., 1990). TSD is a process that depended on a variety of factors which interacted to determine the sex ratio of offspring (Valenzuela, 2004a). On the other hand, this sex ratio variation may affect the evolutionary stability of TSD (Bulmer and Bull, 1982).

In Thailand, although several species of freshwater turtles can be found, an extent of their susceptibility to temperature change is unknown due to the lack of information on gonadal development pattern. The snail-eating turtle, *Malayemys macrocephala*, is a native freshwater turtle commonly found in rice fields in the

central part of Thailand. Previous morphometric analysis on this species suggested a temperature-dependent pattern of sex determination (Keithmaleesatti, 2008). The current study aimed to further examine effect of temperature on gonadal development of *M. macrocephala* using histological analysis in order to confirm the temperature-dependent pattern of sex determination in this turtle species.

6.2 Materials and Methods

6.2.1 Turtle egg collection

Turtle eggs (n=712) were collected from December 2011 to February 2012 from rice fields in Bang Ban district, Phra Nakhon Si Ayutthaya province in central part of Thailand and transported to the laboratory at the Department of Biology, Faculty of Science, Chulalongkorn University. All portions of this research project involving turtle egg collection and animal subjects had been approved by the Chulalongkorn University Animal Care and Use Committee (Protocol Review Number 1323005).

6.2.2 Turtle egg incubation

In the laboratory, eggs were cleaned, weighed and randomly allocated into plastic boxes (19.2 x 28.0 x 5.7 cm) filled with moisted vermiculite (1 part vermiculite : 1 part distilled water, weight : volume). Boxes of the eggs were kept in microprocessor-controlled incubators (Siam Incubators System, Bangkok, Thailand) at three temperatures including 26 °C (n=237), 29 °C (n=237) and 32 °C (n=238). Tray of water was placed inside each incubator to control relative humidity (>80%). Temperature and relative humidity inside the box were monitored using a data logger (HAXO-8, LogTag Recorders, Auckland, New Zealand). Eggs were kept in these condition until hatch.

6.2.3 Embryo collection

Upon emergence, hatching embryos (or hatchlings) were weighed and measured for carapace length (result shows in Chapter V). After the measurement, these embryos were subjected to euthanasia by intraperitoneal injection of

pentobarbital sodium at an overdose (600 mg/kg intraperitoneally). Hatchlings from three temperatures including 26 °C (n=40), 29 °C (n=26) and 32 °C (n=9) were fixed in Davidson's fixative for 24 hours and then preserved in 70% ethanol.

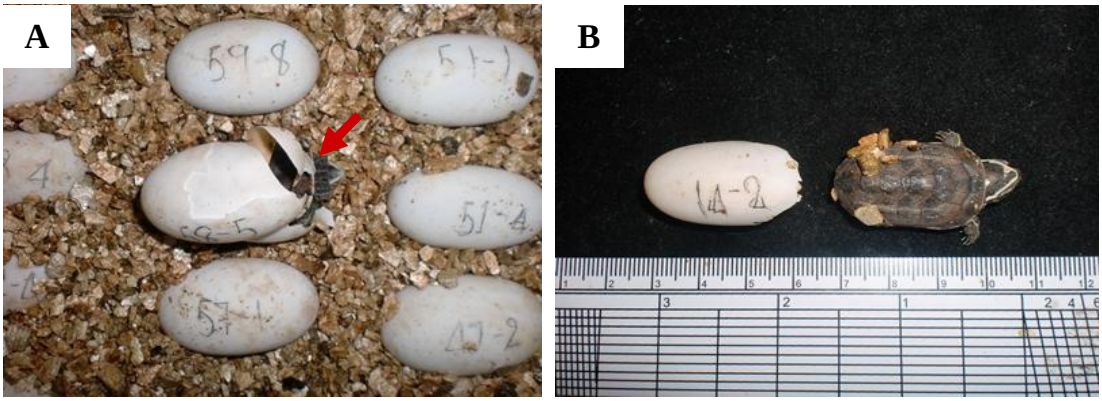


Figure 6.1: The snail-eating turtle hatchling: (A) emergence from the eggshell (arrow) (B) complete emergence of hatchling from the eggshell

6.2.4 Histological examination of gonad

The fixed gonads from each individual were processed following standard histological techniques (Humason, 1979). Gonadal tissues were infiltrated in paraffin, sectioned at 5 μm and stained with periodic acid Schiff (PAS) staining. Histological study was performed by light microscopy. Gonadal development and sex differentiation in *M. macrocephala* were studied in reference to previous report of the snapping turtle, *Chelydra serpentina* (Yntema, 1981). After sex of the embryos was identified by histology, sex ratio of turtle from each temperature was determined.

In addition to sex identification by histological analysis of gonadal structure, testis or ovary from each individual was further examined for stage of development. In case of testis, the stage of development was categorized into 3 stages primarily based on an advancement of seminiferous tubules from medullary region. For ovary,

the stage of development was classified into 3 stages based on progression of germinal epithelium in ovarian cortex (see 6.3.2).

6.2.5 Statistical analyses

For gonadal developmental stage, gonads of hatchlings from each incubating temperature were classified into three arbitrary stages (I, II or III). Then, a 3x3 contingency table was used to determine association between incubating temperatures (26, 29 and 32 °C) and gonadal developmental stages (I, II or III).

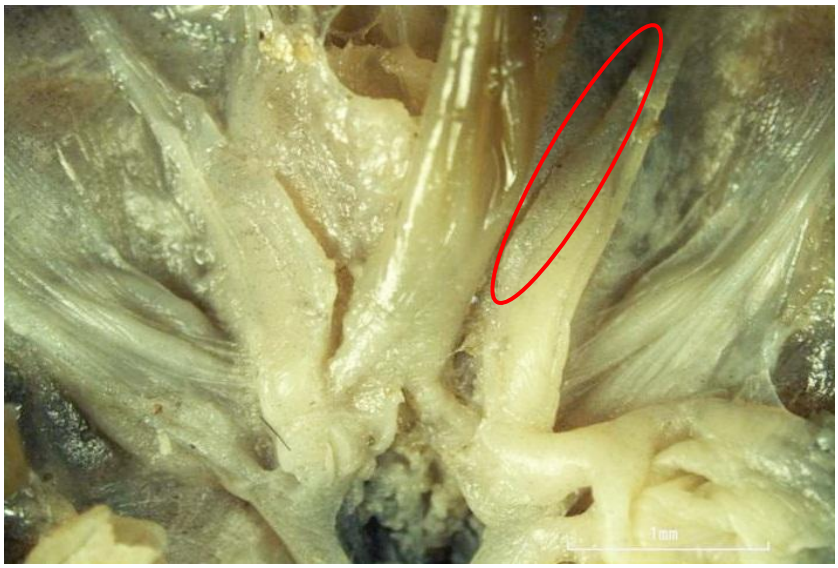
For sex ratio of the hatchlings, *G* statistics for the log-likelihood ratio goodness of fit test (*G* test) with Yates's correction was used to determine whether the sex ratio of turtle from each incubating temperature was best fitted to any expected sex ratio (e.g. 1:1, 1:2 or 2:1, male : female).

A significant association or goodness of fit is reported at $p<0.05$. All statistical procedures used in this study were according to Zar (1998).

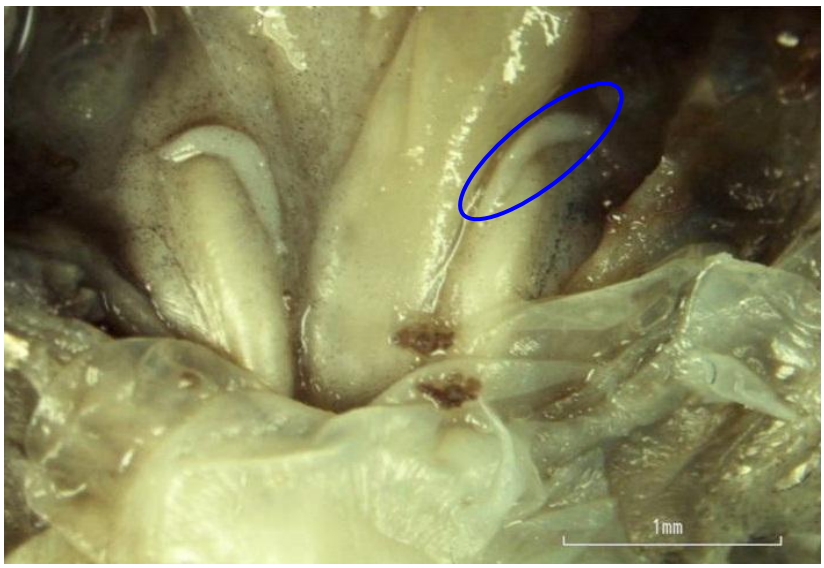
6.3 Results and Discussion

6.3.1 Gross morphology of the gonad of *M. macrocephala*

Since sex of young freshwater turtle is difficult to determine because they lack external sexual dimorphic characteristic (Ceriani and Wyneken, 2008), in this study, sex of the hatchling was initially determined using gross morphology. Upon dissection of the abdominal cavity, an ovary appears as long, thin, oval shaped with a scalloped edge (Figure 6.2), while a testis is fusiform in shape with a smooth edge (Figure 6.3). It is of interest to note the similarity in these structures. Therefore, using gross morphological characteristics of the hatchling gonads alone to differentiate the sex was not enough to verify the sex of embryos.



Figures 6.2: Gross morphological characteristics of female gonad (in red oval line) of *M. macrocephala* hatchling at stage 26.



Figures 6.3: Gross morphological characteristics of male gonad (in blue oval line) of *M. macrocephala* hatchling at stage 26.

6.3.2 Histological analysis of the gonad of *M. macrocephala*

Because of the difficulty in using the gross morphological characteristics of the gonads to differentiate the sex of the turtle hatchlings, histological examination was used to confirm the sex of embryos. In freshwater turtle, gonad is generally fully differentiated into testes or ovaries at hatching stage. Histological study of gonadal development in *M. macrocephala* was thus studied based on a previous report in the snapping turtle, *Chelydra serpentina* (Yntema, 1981).

The developing testis can be divided into 2 zones including cortex and medulla. Histological structure of the testicular cortex was poorly developed whereas the medulla contained germ cells in mesenchyme tissue. Testicular development of the male *M. macrocephala* hatchling could be divided into three stages as described below.

- Stage I: The testicular cortex was covered with connective tissue capsule. The testicular medulla displayed wide cords of primordial germ cells or tubes (Figure 6.4 A, B).
- Stage II: The testicular medulla was branched out and transformed into immature seminiferous cords containing germ cells (Figure 6.4 C, D).
- Stage III: The testicular medulla contained many germ cells and transformed into immature seminiferous tubules (Figure 6.4 E, F).

The developing ovary can be divided into 2 zones including cortex and medulla. Histological structure of the ovarian cortex was advanced whereas the medulla consisted mostly of diffused mesenchyme. This characteristics is a marker of ovary. Ovarian development of the female *M. macrocephala* hatchling could be classified into three stages as described below.

- Stage I: The ovarian cortex was thin containing germ cells and separated from the internal medulla. The PAS-positive line can be seen separating between these 2 layers. The ovarian medulla consisted mostly of diffused mesenchyme (Figure 6.5 A, B).
- Stage II: The ovarian cortex become thicker containing numerous germ cells (Figure 6.5 C, D).
- Stage III: The ovarian cortex was well developed containing many germ cells and primary oocytes (Figure 6.5 E, F).

Figure 6.4: Photomicrographs of testes of *M. macrocephala* hatchling at different gonadal developmental stages (PAS stain).

(A) Testicular tissue at low magnification showed overall structure of the testis at Stage I.

(B) High magnification of the testis showed primordial germ cells (black arrows) in the medulla (M).

(C) Testicular tissue at low magnification showed overall structure of the testis at Stage II.

(D) High magnification of the testis showed primary sex cords (asterisks).

(E) Testicular tissue at low magnification showed overall structure of the testis at Stage III.

(F) High magnification of the testis showed seminiferous tubules (asterisks).

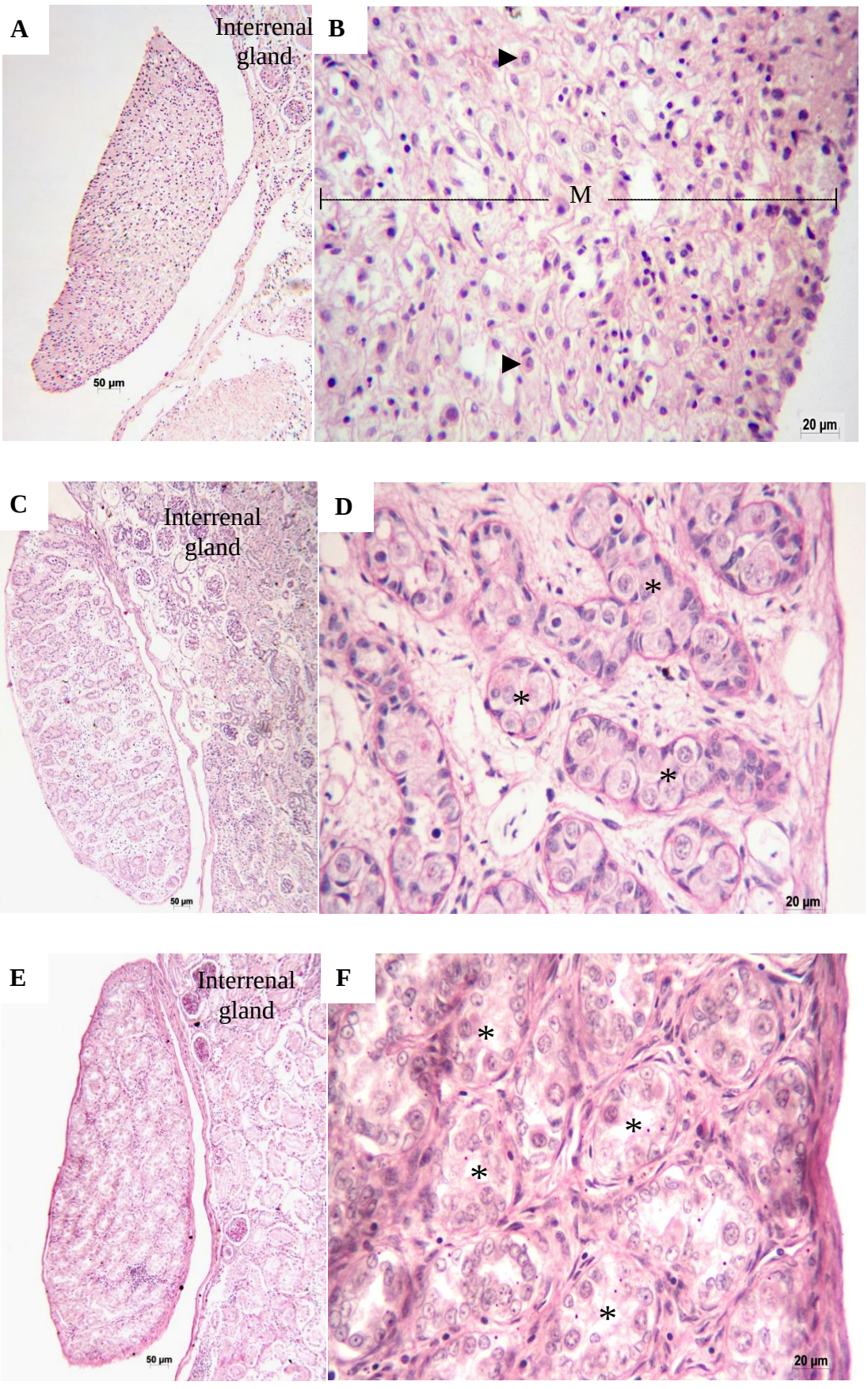

A
Interrenal gland
50 µm
B
M
20 µm
C
Interrenal gland
50 µm
D
20 µm
E
Interrenal gland
50 µm
F
20 µm

Figure 6.5: Photomicrographs of ovaries of *M. macrocephala* hatchling at different gonadal developmental stages (PAS stain).

(A) The ovarian tissue at low magnification showed overall structure of the ovaries at Stage I.

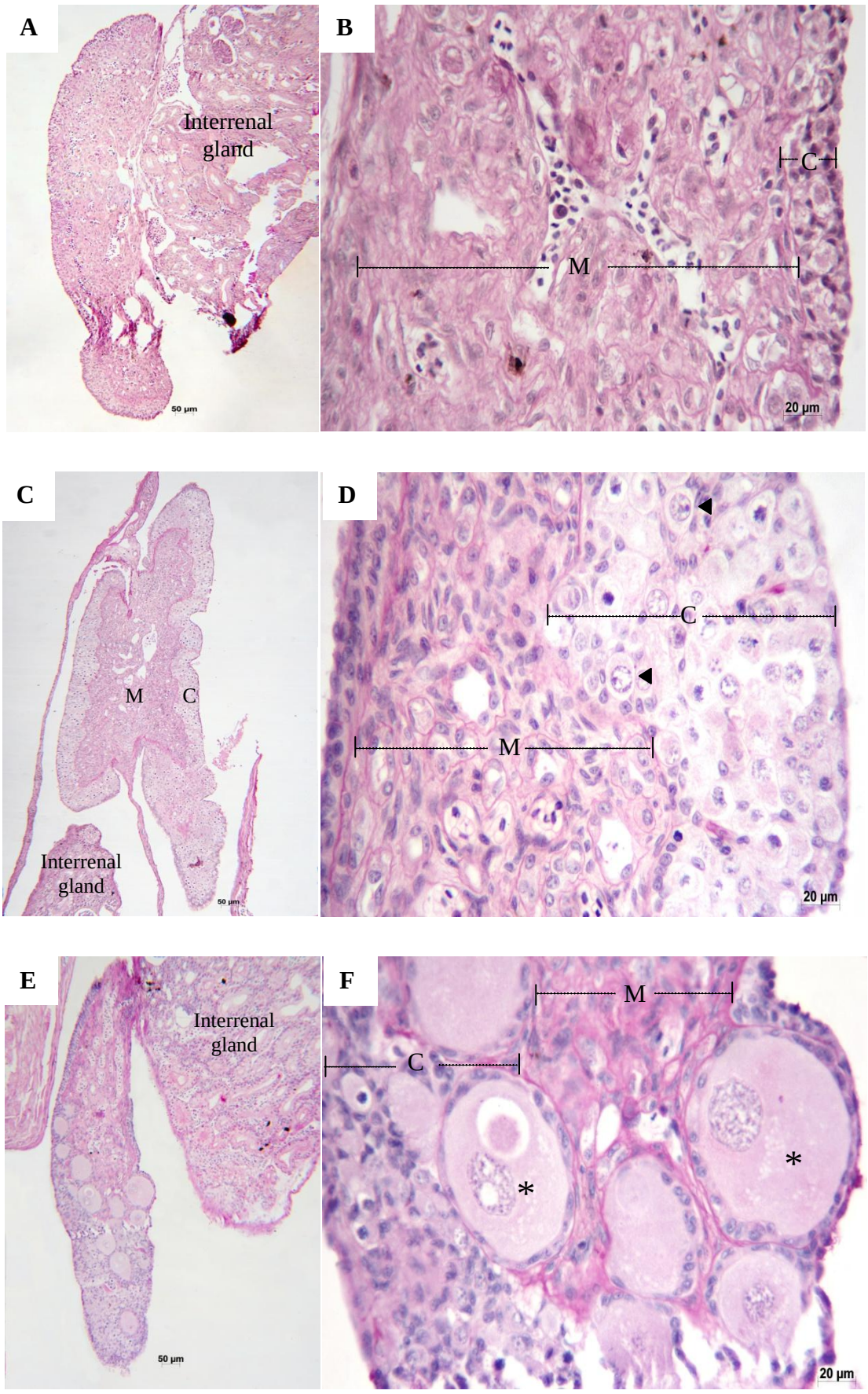
(B) High magnification of the ovary showed primordial germ cells (arrows) in the thin cortex (C).

(C) The ovarian tissue at low magnification showed overall structure of the ovary at Stage II. Medulla (M) and cortex (C) are discriminated.

(D) High magnification of the ovary showed germ cells (arrows) are present in the thick cortex (C).

(E) The ovarian tissue at low magnification showed overall structure of the ovary at Stage III.

(F) High magnification of the ovary showed primary oocytes in the cortex (asterisks).


A
Interrenal gland
50 µm
B
C
M
20 µm
C
M
C
Interrenal gland
50 µm
D
C
M
20 µm
E
Interrenal gland
50 µm
F
M
C
*
*
20 µm

6.3.3 Effect of temperature on gonadal developmental stage

Stage of testicular development of male *M. macrocephala* was determined by histological analysis. In male turtle, testicular development was more advanced at high temperature (32 °C) than other temperatures (26 °C and 29 °C) as evidenced from the higher proportion of Stage III testis in turtles incubated at 32 °C (Figure 6.6). As a result, the 3 x 3 contingency table analysis (Table 6.1) shows a significant association between incubating temperature and the stage of testicular development of *M. macrocephala* (χ^2 =10.14, d.f. = 4, $p<0.05$).

Stage of ovarian development of female *M. macrocephala* was determined by histological analysis. In female turtle, developmental stage of the ovaries was more advanced at pivotal temperature (29 °C) than other temperature (26 °C and 32 °C; Figure 6.7). The 3 x 3 contingency table analysis (Table 6.2) shows a significant association between incubating temperature and the stage of ovarian development of *M. macrocephala* (χ^2 =21.79, d.f. = 4, $p<0.05$).

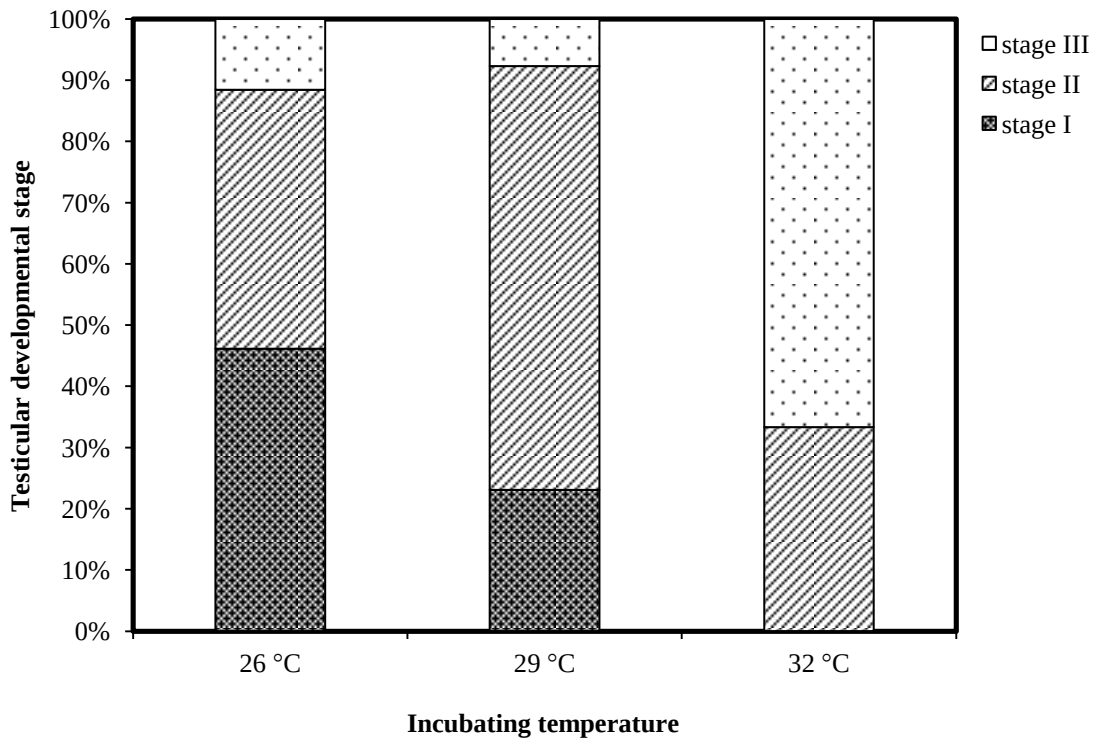


Figure 6.6: Proportions of *M. macrocephala* hatchling with different testicular developmental stage in each incubating temperature.

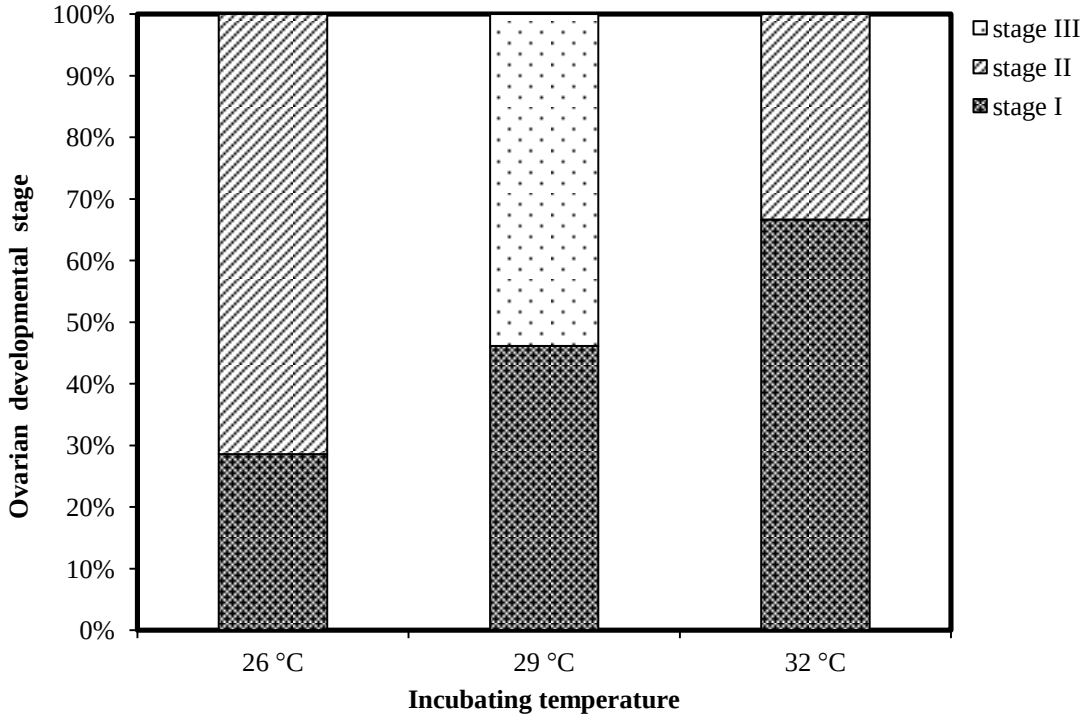


Figure 6.7: Proportions of *M. macrocephala* hatchling with different ovarian developmental stage in each incubating temperature.

Table 6.1: Contingency table analysis for association between incubating temperature and the stage of testicular development of *M. macrocephala*

Testicular development		Temperature			Total
		26 °C	29 °C	32 °C	
Stage I	Observed (O)	12	3	0	15
	Expected (E)	9.29	4.64	1.07	
	$(0-E)^2/E$	0.79	0.58	1.07	
Stage II	Observed (O)	11	9	1	21
	Expected (E)	13	6.5	1.5	
	$(0-E)^2/E$	0.31	0.96	0.17	
Stage III	Observed	3	1	2	6
	Expected	3.71	1.86	0.43	
	$(0-E)^2/E$	0.14	0.40	5.73	
Theoretical value: $\chi^2 = 9.49$, d.f. = 4, p=0.05					
Calculated value: $\chi^2 = 10.14$					

Table 6.2: Contingency table analysis for association between incubating temperature and the stage of ovarian development of *M. macrocephala*

Ovarian development		Temperature			Total
		26 °C	29 °C	32 °C	
Stage I	Observed (O)	4	6	4	14
	Expected (E)	5.94	5.52	2.55	
	$(0-E)^2/E$	0.63	0.04	0.82	
Stage II	Observed (O)	10	0	2	12
	Expected (E)	5.09	4.73	2.18	
	$(0-E)^2/E$	4.74	4.73	0.01	
Stage III	Observed	0	7	0	7
	Expected	2.97	2.75	1.27	
	$(0-E)^2/E$	2.97	6.57	1.27	

Theoretical value: $\chi^2 = 9.49$, d.f. = 4, p=0.05

Calculated value: $\chi^2 = 21.79$

6.3.4 Effect of temperature on sex ratio (*G*-statistics)

M. macrocephala hatched from eggs incubated at three different temperatures were examined for sex by histological examination of the gonad. Sex ratio from each incubating temperature was shown in Figure 6.8 and Table 6.3. The result showed that low incubating temperature (26 °C) resulted in the highest proportion of male turtle whereas high temperature (32 °C) showed the highest proportion of female turtle. The result also showed that pivotal temperature (29 °C) showed the equal proportion of male and female turtles.

G tests were performed to test for goodness of fit at three expected sex ratios of *M. macrocephala* (male-biased sex ratio, 1:1 sex ratio and female-biased sex ratio). In each temperature, the best fitted sex ratio is determined from the *G* value that must meet the following two assumptions: 1) *G* value must be lower than the theoretical χ^2 value ($\chi^2_{0.05, \text{d.f. } 1} = 3.841$), and 2) the lowest *G* value (Zar, 1998). The result indicated that incubation at low temperature (26 °C) produced male-biased sex ratio, while incubation at high temperature (32 °C) produced female-biased sex ratio. Incubation at 29 °C resulted in 1 male : 1 female sex ratio confirming that this incubating temperature is a pivotal temperature of *M. macrocephala*. The result in this study is similar to study in *Chrysemys picta* (Bull et al., 1982) and *Chinemys reevesii* which showed pivotal temperature in the range of 28-30 °C (Du et al., 2007).

Many species of turtle are reported to have TSD (reviewed in Ewert et al., 2004). Incubation temperature play important role in determining sex ratio of freshwater turtle population (Bull et al., 1980; Bull et al., 1990). Among 79 species of turtles examined, 64 species of turtle in the world are known to have TSD (Ewert et al., 2004). For the snail-eating turtle, previous studies of *M. macrocephala*

from Phra Nakhon Si Ayutthaya using morphometric analysis (Keithmaleesatti, 2008) predicted that sex ratio of hatchlings showed a temperature-dependent pattern (TSD). The current research using histological analysis confirmed the pattern of temperature-dependent sex determination of this species. This result provides a new record of TSD in this tropical freshwater turtle species (Ewert et al., 2004).

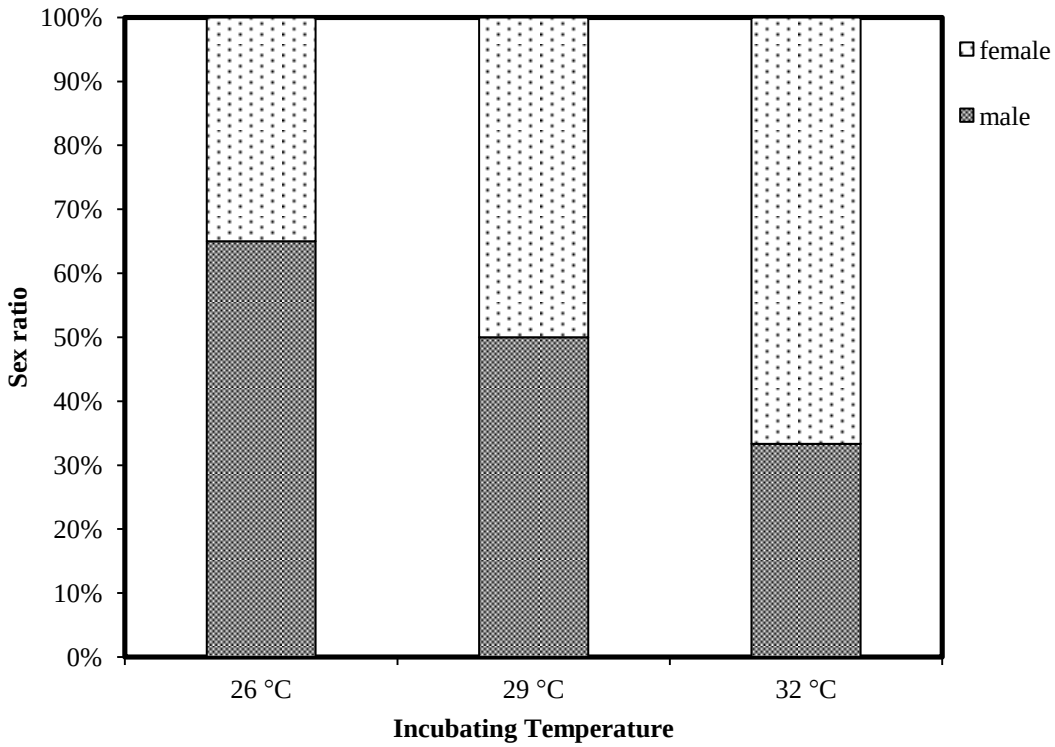


Figure 6.8: Sex ratios of *M. macrocephala* hatched from eggs incubated at three different temperatures

Table 6.3: *G* statistics for the log-likelihood ratio goodness of fit test of sex ratio of *M. macrocephala* hatched from eggs incubated at three different temperatures

Temperature	Sex ratio (male : female)		
	2 : 1	**1 : 1**	**1 : 2**
26 °C (n=40)	0.1* ($\chi^2_{0.05, d.f. 1} = 3.841$)	3.6 ($\chi^2_{0.05, d.f. 1} = 3.841$)	12.91 ($\chi^2_{0.05, d.f. 1} = 3.841$)
29 °C (n=26)	3.24 ($\chi^2_{0.05, d.f. 1} = 3.841$)	0.0* ($\chi^2_{0.05, d.f. 1} = 3.841$)	3.24 ($\chi^2_{0.05, d.f. 1} = 3.841$)
32 °C (n=9)	4.5 ($\chi^2_{0.05, d.f. 1} = 3.841$)	1.0 ($\chi^2_{0.05, d.f. 1} = 3.841$)	0.0* ($\chi^2_{0.05, d.f. 1} = 3.841$)

Remarks: The best fitted sex ratio (*) is determined from the lowest *G* value that must be lower than the theoretical χ^2 value.

6.4 Conclusion

Many species of oviparous reptiles, including crocodilians, turtles and lizards have shown temperature-dependent sex determination pattern. In this study, histological analysis of gonad of *M. macrocephala* incubated at different temperature confirmed that the snail-eating turtle also exhibits a temperature-dependent sex determination. Incubation at low temperature (26 °C) produced male-biased sex ratio. Incubation at high temperature (32 °C) produced female-biased sex ratio. Incubation at the pivotal temperature (29 °C) resulted in 1 male : 1 female sex ratio. Furthermore, the result extended prior observations that incubating temperature was an important variable affecting differentiation of gonads into ovaries or testes as well as degree of development of the gonad in *M. macrocephala*. This information could be used to assess the impact of global trend of temperature change and potential mitigation measure to reduce this impact on the freshwater turtle in order to conserve their natural populations.

CHAPTER VII

GENERAL CONCLUSION AND RECOMMENDATIONS

7.1 General conclusion

Increase in average temperature that gradually warms the earth can change both physical and biological environment and affects survival of organisms. Organisms that cannot adjust oneself to suit with the changed environment may die down and become extinct (Walther et al., 2002). Reptiles, especially freshwater turtles, are considered as susceptible species since their sex determination is dependent of incubating temperature. Previous studies on *Malayemys macrocephala* predicted that sex ratio showed a temperature-dependent pattern and suggested additional study on gonadal histology to confirm this pattern (Keithmaleesatti, 2008). This research thus aimed to verify these findings with additional studies on the nesting biology, a series of developmental stage, and effects of incubating temperature on somatic and gonadal development of *M. macrocephala*.

Initially, nesting biology of *M. macrocephala* in rice field at Bang Ban district, Phra Nakhon Si Ayutthaya province, the central part of Thailand was examined. Previous records showed that an onset of nesting season of *M. macrocephala* in this area always occurred in November and last until April. Field observation showed that an onset of the 2011-2012 nesting season of the turtle was delayed for almost month due to lack of nesting ground, possibly as a result of severe flooding in central part of Thailand during October 2011 to mid-January 2012. However, nesting activity was resumed and continued at a usual rate afterward. Clutch size of the turtle found in this study (3-9 eggs) was similar to previous record of *Malayemys* turtles. Average nest temperature ranged from 23.13 to 32.02 °C indicated potential exposure to wide range

of temperature. Furthermore, new records of abnormality in turtle egg were documented in a clutch of turtle eggs found during this study period.

Next, developmental stage of *M. macrocephala* was examined from embryos derived from eggs incubated at 29 °C. Morphological characters of embryos were examined in reference to the widely used stage of development of *Chelydra serpentina* (Yntema; 1968) and staging criteria of *Pelodiscus sinensis* (Tokita and Kuratani, 2001). The results of this study showed that a series of developmental stages of *M. macrocephala*, the native and the most common freshwater turtles in Thailand, can be established with 21 discrete embryonic stages (stages 6 through 26). Using this developmental stage as parameters of growth, it was found that the developmental stage of *M. macrocephala* was comparable with the reference species.

After that, effects of temperature on somatic development of *M. macrocephala* were studied at three different temperatures (26, 29 and 32 °C). The incubation periods, or time required for egg to hatch, of turtle eggs incubated at three different temperatures ranged from 78-150 days. The result indicated that there was no significant difference in incubation periods among temperatures. Based on somatic development, it is apparent that incubating temperature could affect growth and development of turtle embryos. Incubation at high temperature (32 °C) resulted in significantly more rapid growth and development than other temperature (26 °C and 29°C). However, incubation at high temperature seemed to be harmful to embryo since higher incidence of deformities was found.

Finally, effects of temperature on gonadal development of *M. macrocephala* were studied at these three incubating temperatures (26, 29 and 32 °C). Histological analysis of gonad of *M. macrocephala* incubated at different temperature confirmed

prior observations and concluded that *M. macrocephala* exhibits a temperature-dependent sex determination (TSD). Incubation at low temperature (26 °C) produced male-biased sex ratio, while incubation at high temperature (32 °C) produced female-biased sex ratio, and incubation at the pivotal temperature (29 °C) resulted in 1 male: 1 female sex ratio. Furthermore, the result extended prior observations that the incubating temperature could affect differentiation of gonads into ovaries or testes as well as degree of development of the gonad of *M. macrocephala*.

Overall, the results indicated that incubating temperature is an important variable affecting both somatic and gonadal development of *M. macrocephala*. This information could be used to assess the impact of global trend of temperature change and potential mitigation measure to reduce this impact on the freshwater turtle in order to conserve their natural populations.

7.2 Recommendations

Based on results of this study, it is apparent that *M. macrocephala* exhibits a temperature-dependent sex determination (TSD). To use this species as animal model for TSD, it is suggested that information on transitional range of temperature and critical period for sex determination should be further examined (Valenzuela, 2004a).

Adverse effects of high temperature on somatic and gonadal development of *M. macrocephala* were obvious. Observations in the field also revealed that more than 18% of turtle nests had high nest temperature (>32 °C, Appendix A). To minimize effects of temperature in natural population, it is recommended that recreation of a more tolerable habitat (Mrosovsky and Godfrey, 2010), i.e. more tree shade to cool the breeding ground, should be implemented in the turtle nesting area.

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APPENDICES

APPENDIX A

Characteristics of *M. macrocephala* nests at Bang Ban district, Phra Nakhon Si Ayutthaya province, central part of Thailand

Characteristics of *M. macrocephala* nests in rice fields at Bang Ban district, Phra Nakhon Si Ayutthaya province

Nesting areas was usually located near water such as wet paddy ridge and edge of canal. The turtle nest was a u-shape hole with cover made from soil. It is likely that the nesting turtle used soil in vicinity of the nest for covering as it contained live plant, dry plant or no plant materials (Figures A1 A-D).

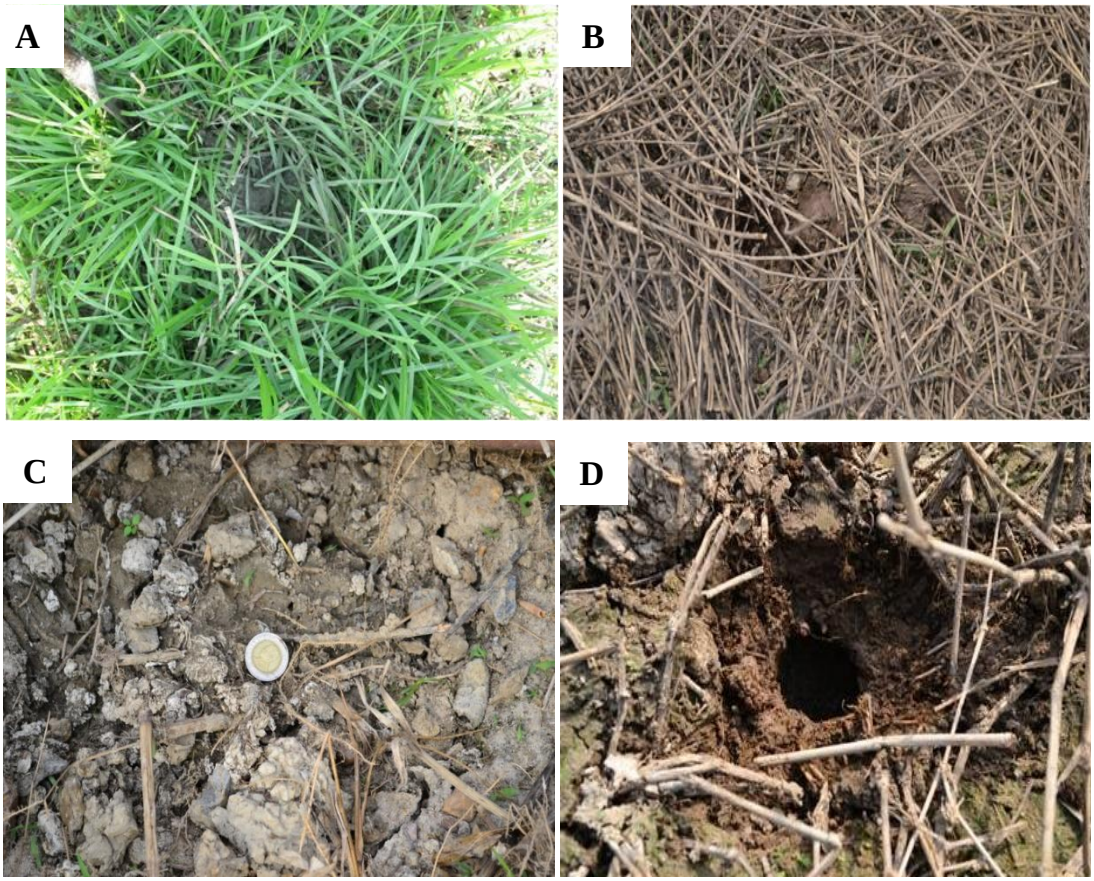

Figure A1 The cover and hole of the snail-eating turtle nest (A) the hole with cover made from soil with live plant materials (B) the hole with cover made from soil with dry plant materials (C) the hole with cover made from plain soil (D) hole of the snail-eating turtle nest

Survey for turtle nest was found to be very challenging. The only clue for turtle nest was its cover. Based on these current surveys, nest cover could be similar to surrounding soil (Figure A2 A) or different from surrounding soil (Figures A2 B).

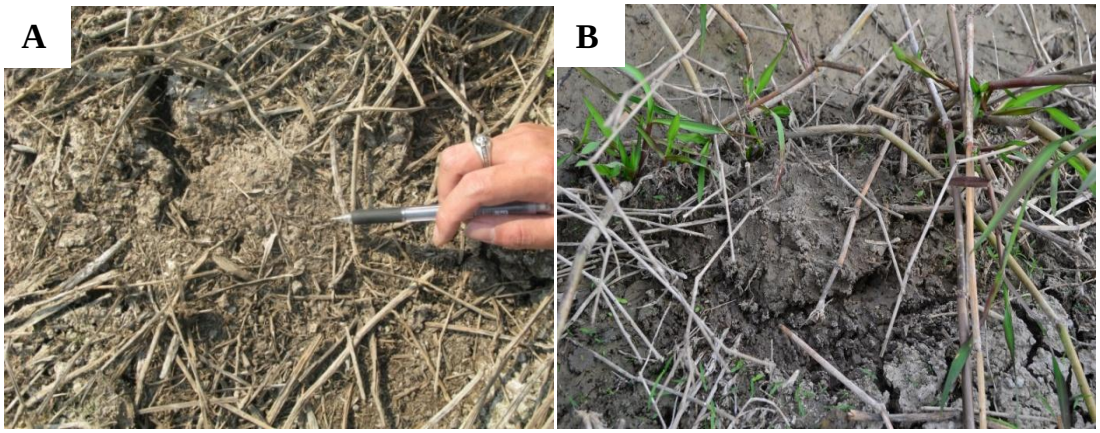

Figure A2 The turtle nest cover: (A) similar to surrounding soil (B) different from surrounding soil

Although the turtle was usually found near water, nest site selection seemed to be less dependent on soil moisture content as the nest soil was found to be wide ranged in water content from wet to dry soil (Figures A3 A-B).

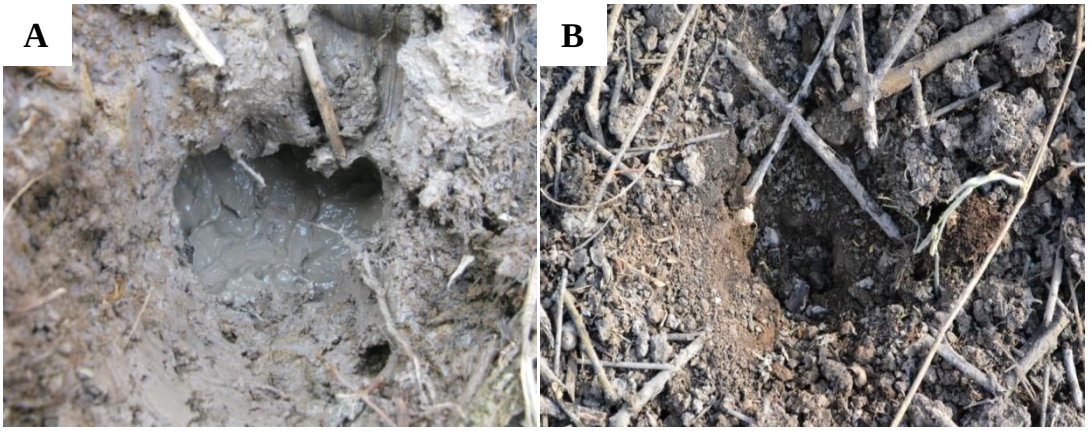

Figure A3 The nest soil: (A) wet soil (B) dry soil

Average nest soil temperature during the surveys was 28.47±3.80 °C with the range of 20.50 °C to 36.50 °C. Majority of turtle nests (71 out of 126 clutches) showed nest soil temperature in the range of 26-32 °C (Table A1). Therefore incubation temperatures of 26-32 °C were used in this study (Chapter IV, V and VI). However, it is of interest to note that 25.4% of turtle nest (35 out of 126 clutches) had nest soil temperature lower than 26 °C and 18.2% of turtle nest (23 out of 126 clutches) had nest soil temperature higher than 32 °C.

Table A1 Range of soil temperature in the nest of *Malayemys macrocephala* at Bang Ban district, Phra Nakhon Si Ayutthaya province, Thailand during 2011-2012

Range of nest soil temperature	**Number of Clutch**
≤ 26	32
26.01-28.00	32
28.01-30.00	20
30.01-32.00	19
≥ 32	23

APPENDIX B

Average temperature and relative humidity from a data logger

at three incubating temperatures (26, 29 and 32 °C)

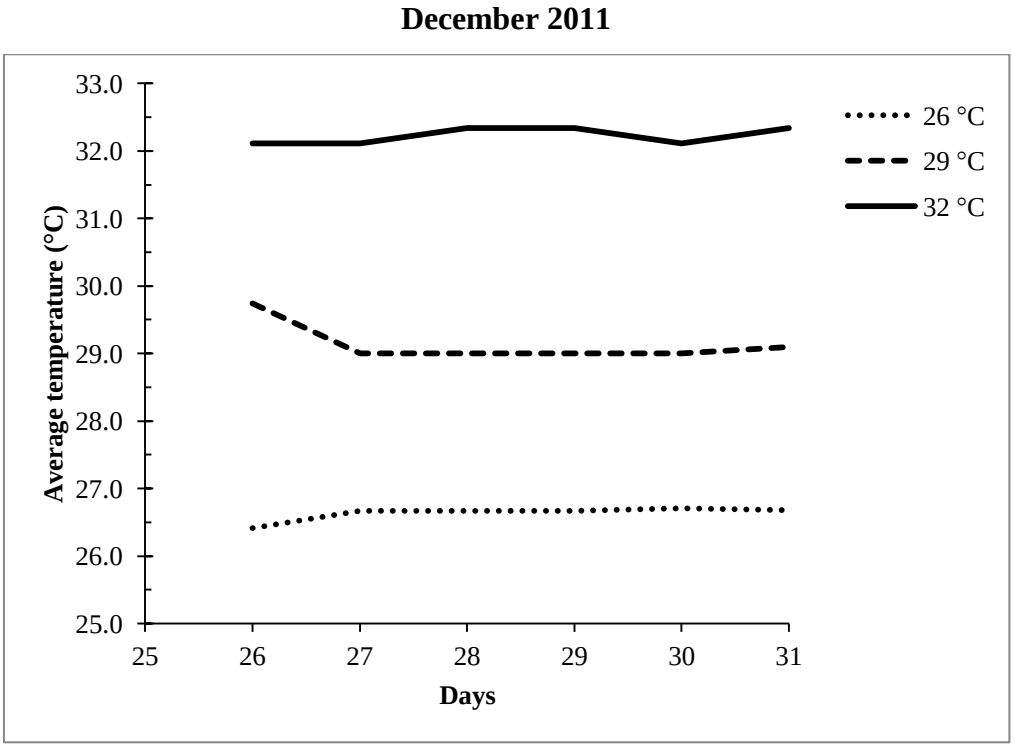

Figure B1 Average daily temperature in microprocessor-controlled incubators at different incubating temperatures in December 2011.

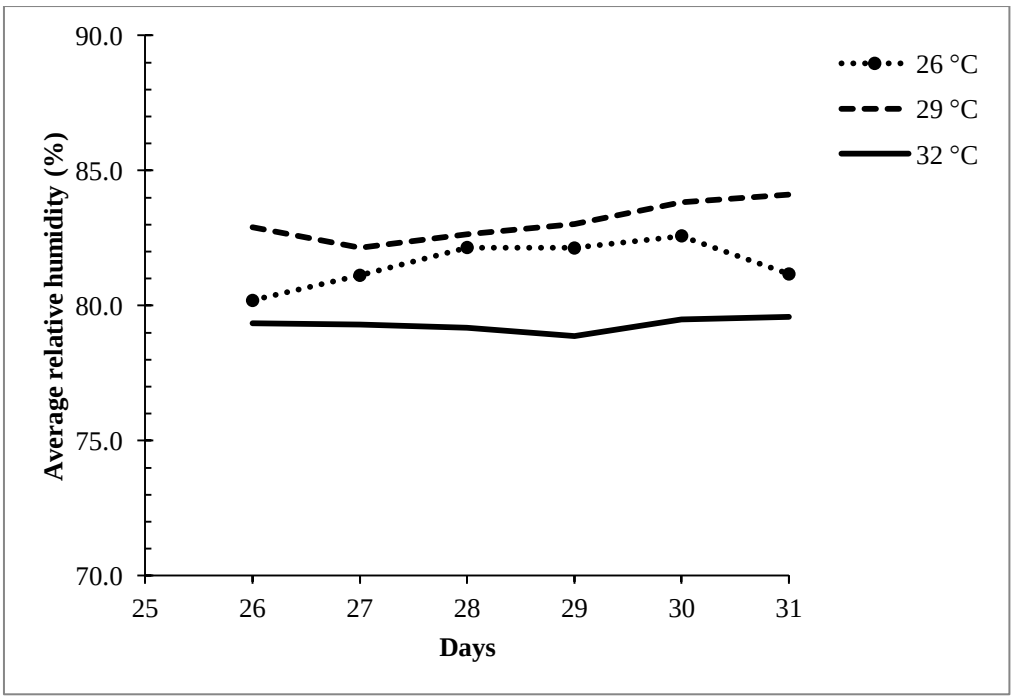

Figure B2 Average daily relative humidity in microprocessor-controlled incubators at different incubating temperatures in December 2011.

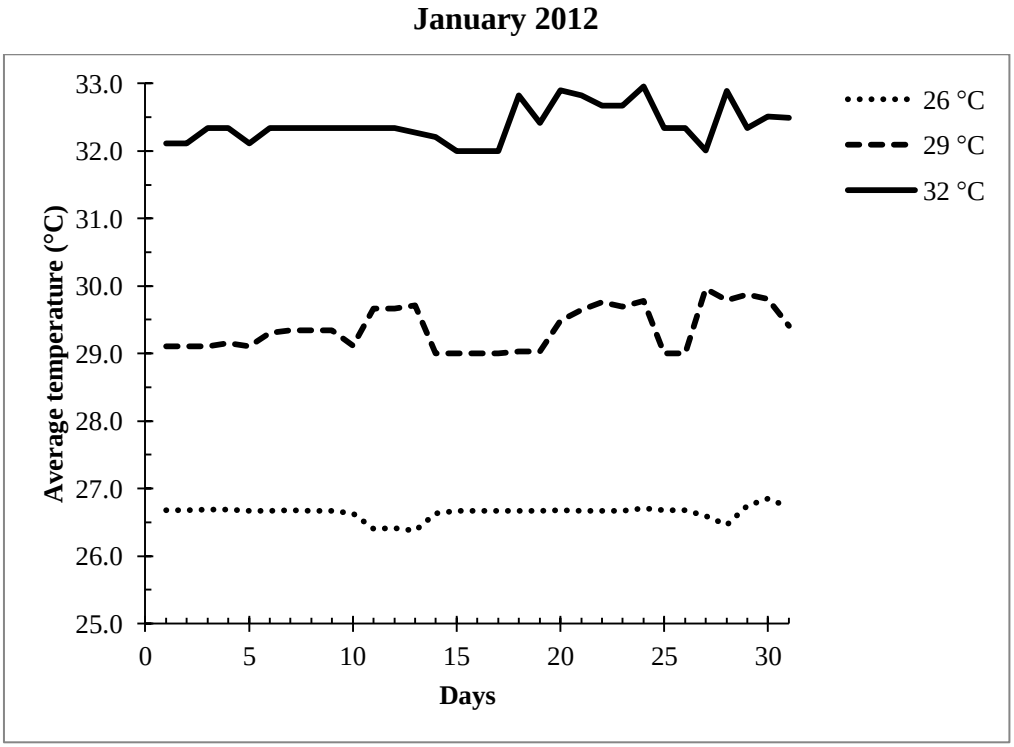

Figure B3 Average daily temperature in microprocessor-controlled incubators at different incubating temperatures in January 2012.

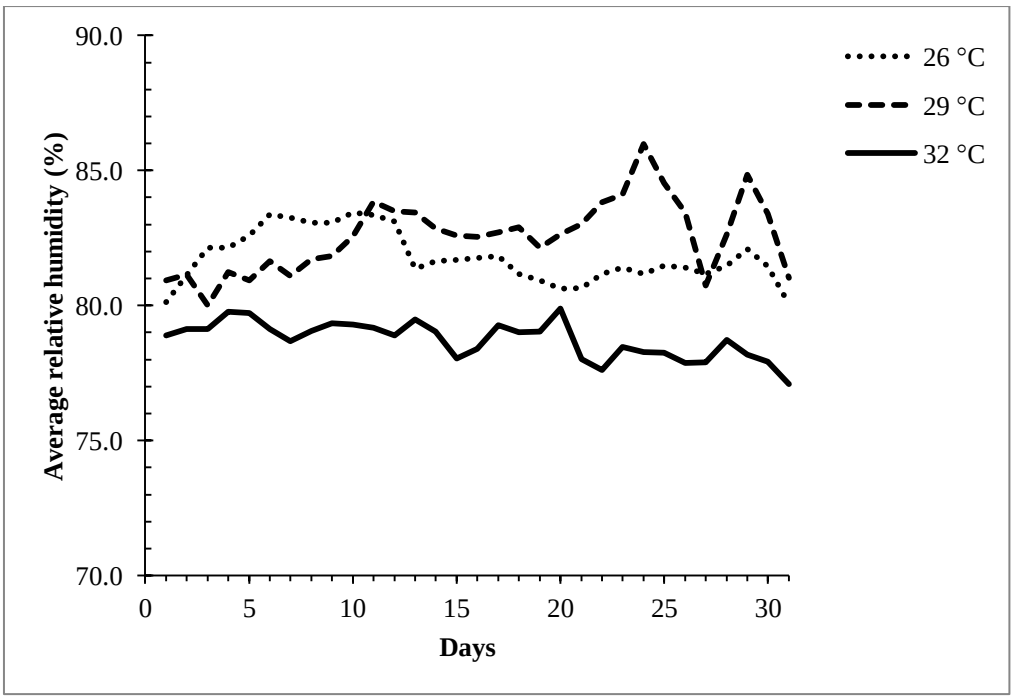

Figure B4 Average daily relative humidity in microprocessor-controlled incubators at different incubating temperatures in January 2012.

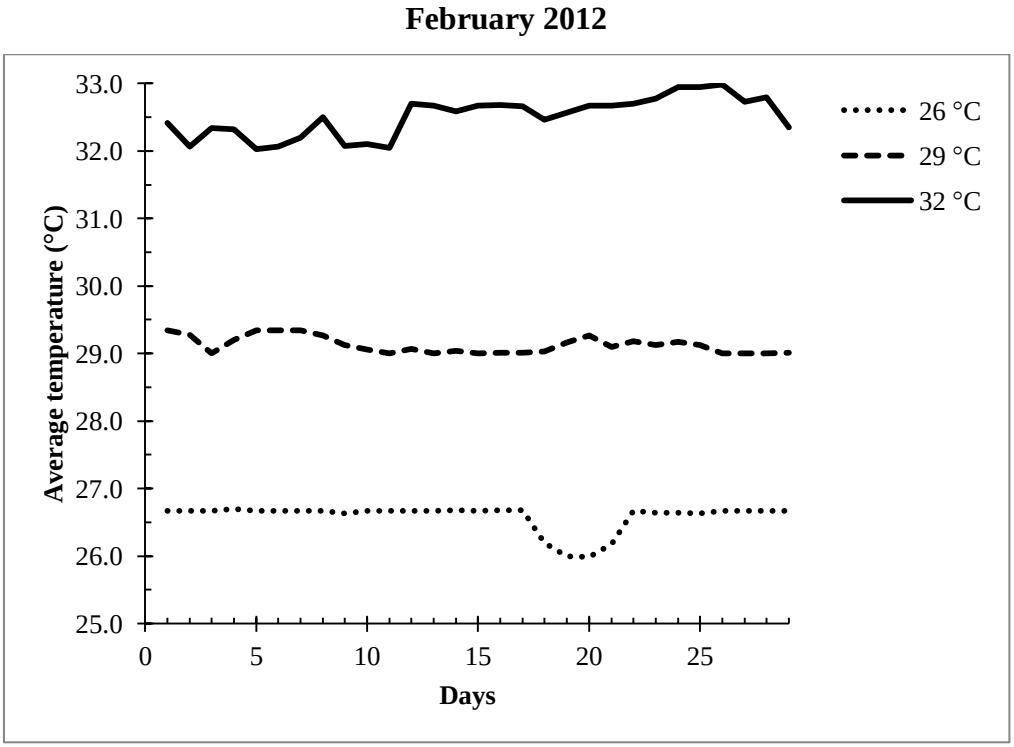

Figure B5 Average daily temperature in microprocessor-controlled incubators at different incubating temperatures in February 2012.

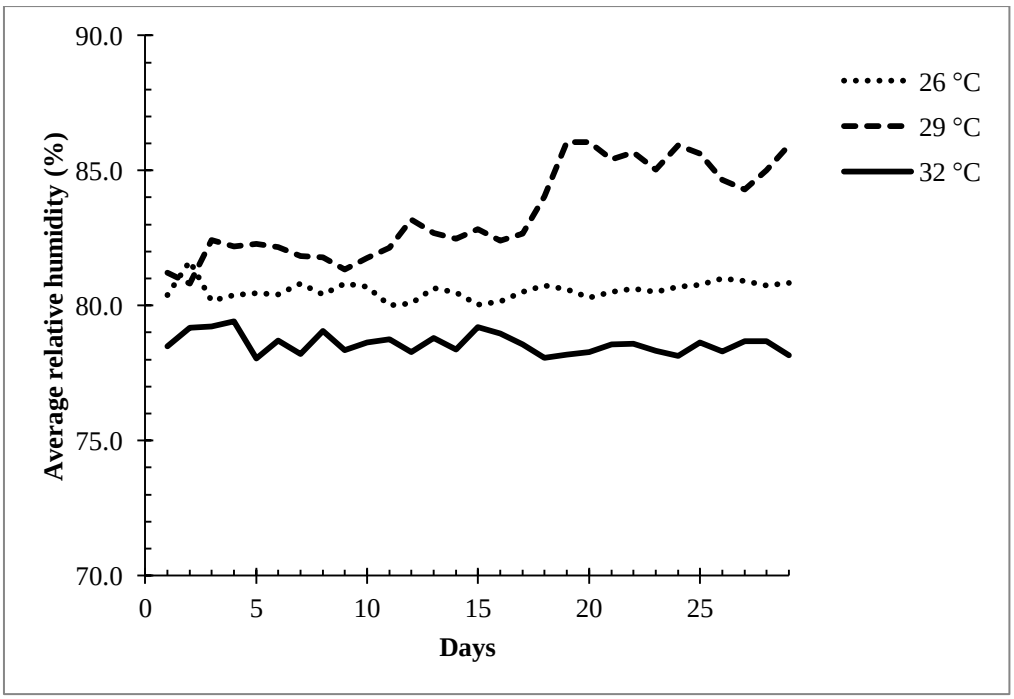

Figure B6 Average daily relative humidity in microprocessor-controlled incubators at different incubating temperatures in February 2012.

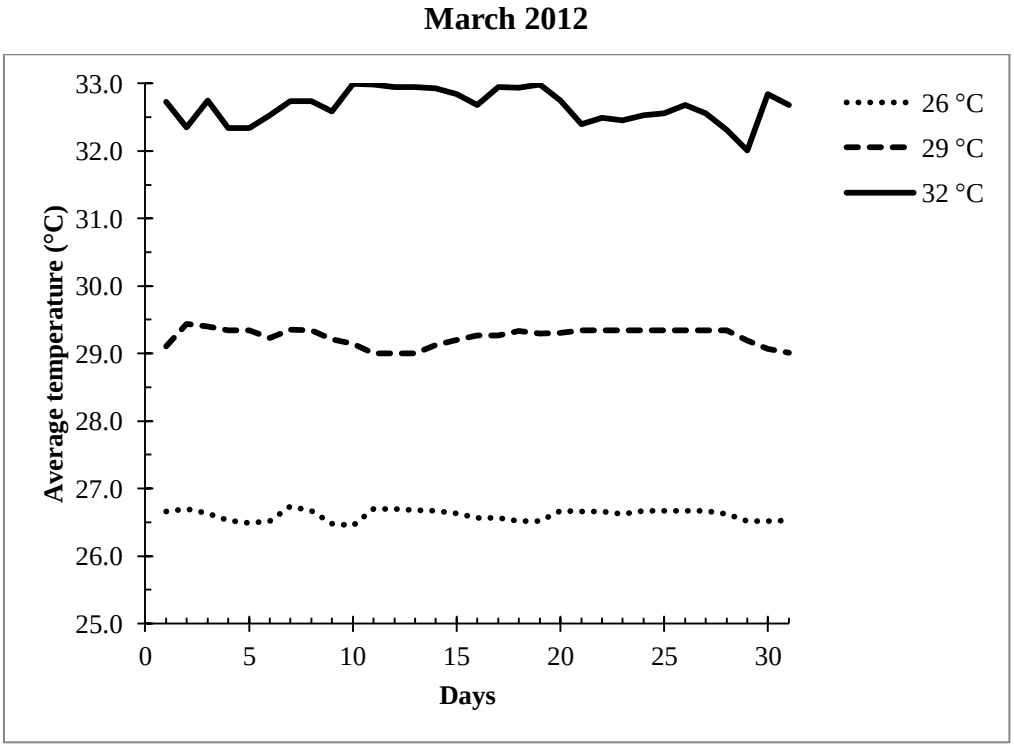

Figure B7 Average daily temperature in microprocessor-controlled incubators at different incubating temperatures in March 2012.

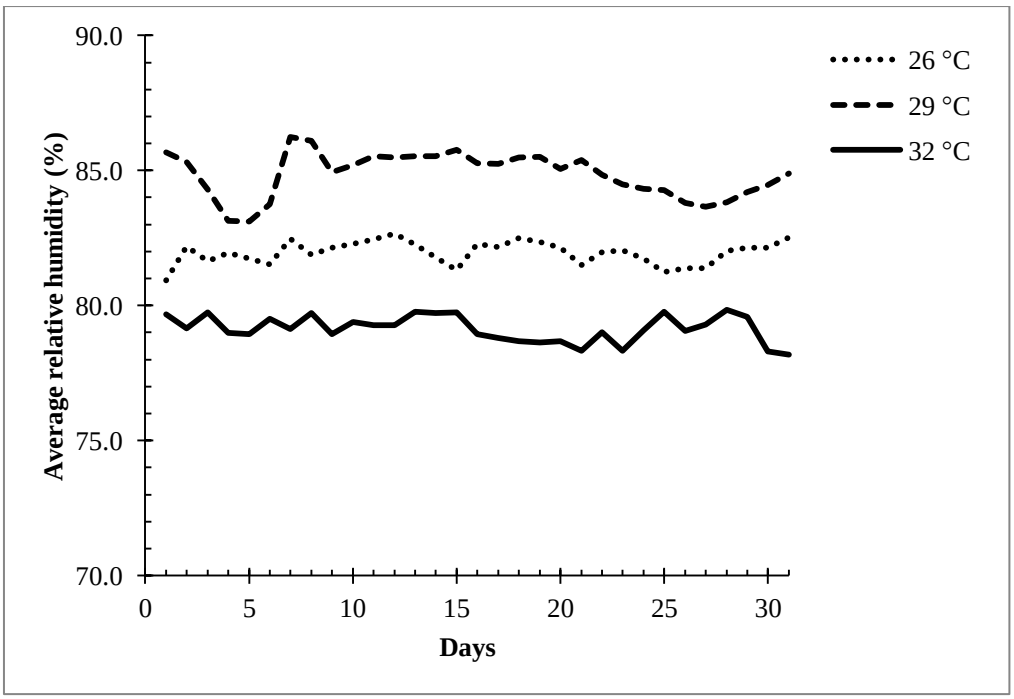

Figure B8 Average daily relative humidity in microprocessor-controlled incubators at different incubating temperatures in March 2012.

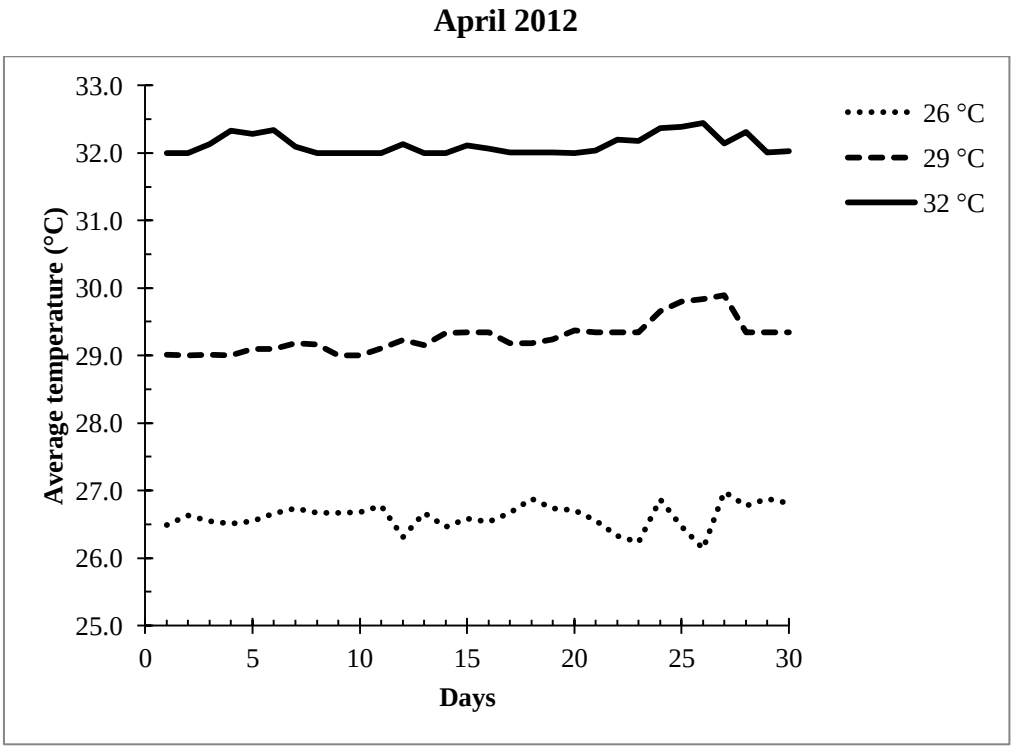

Figure B9 Average daily temperature in microprocessor-controlled incubators at different incubating temperatures in April 2012.

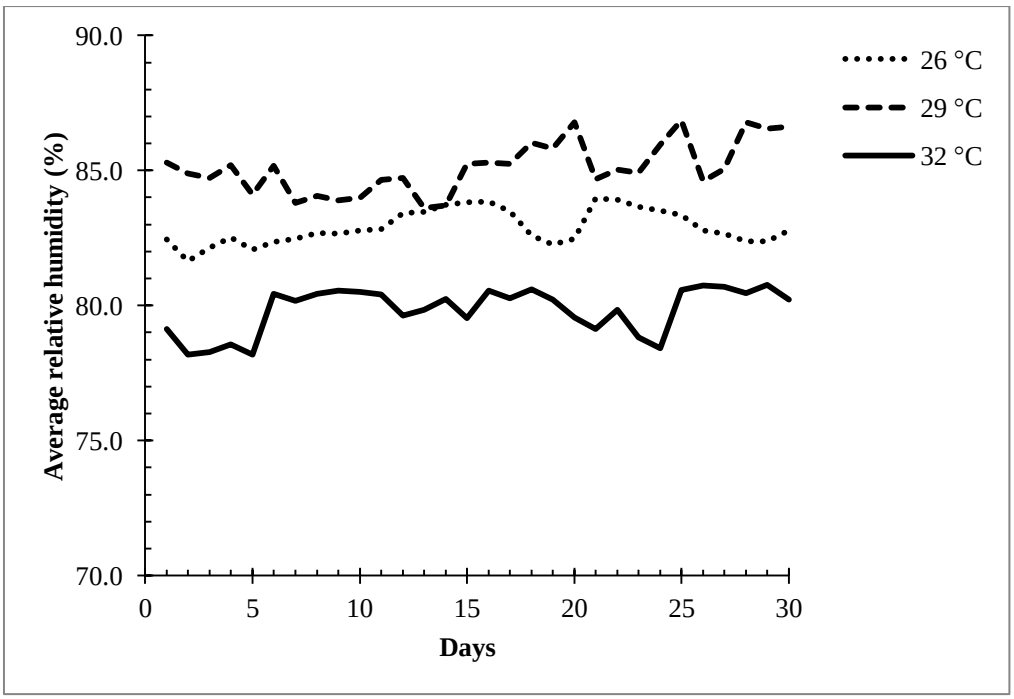

Figure B10 Average daily relative humidity in microprocessor-controlled incubators at different incubating temperature in April 2012.

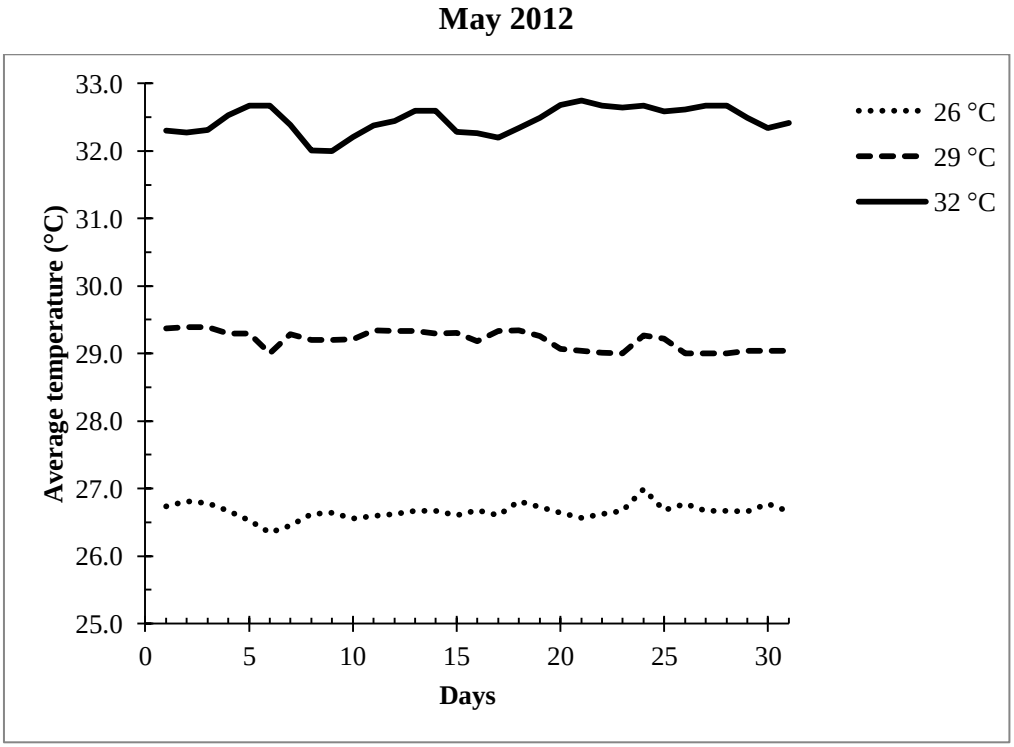

Figure B11 Average daily temperature in microprocessor-controlled incubators at different incubating temperatures in May 2012.

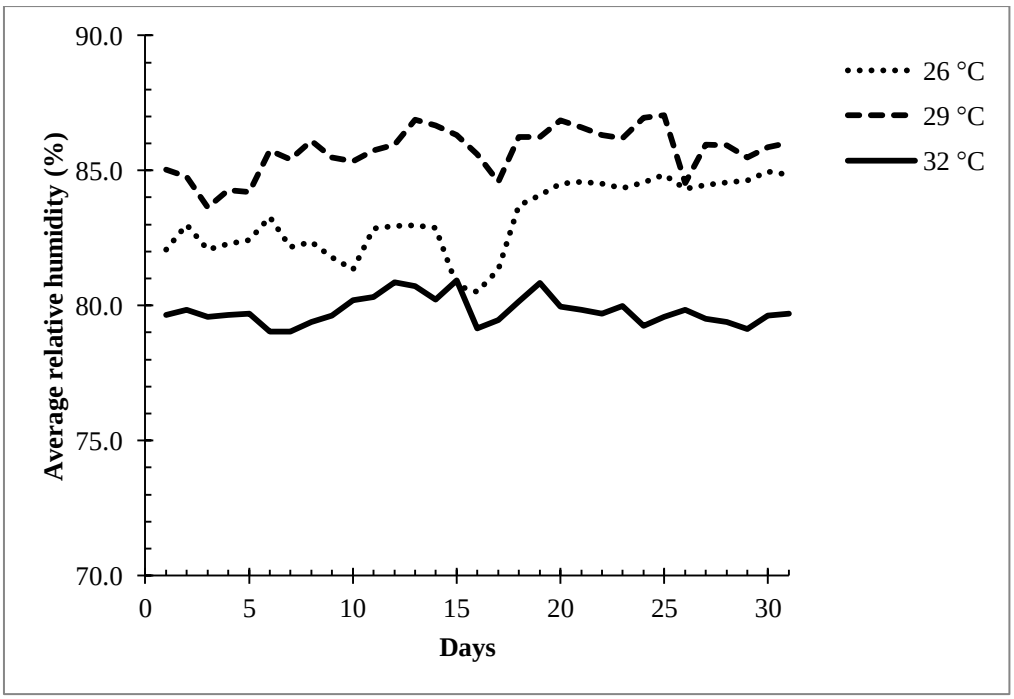

Figure B12 Average daily relative humidity in microprocessor-controlled incubators at different incubating temperatures in May 2012.

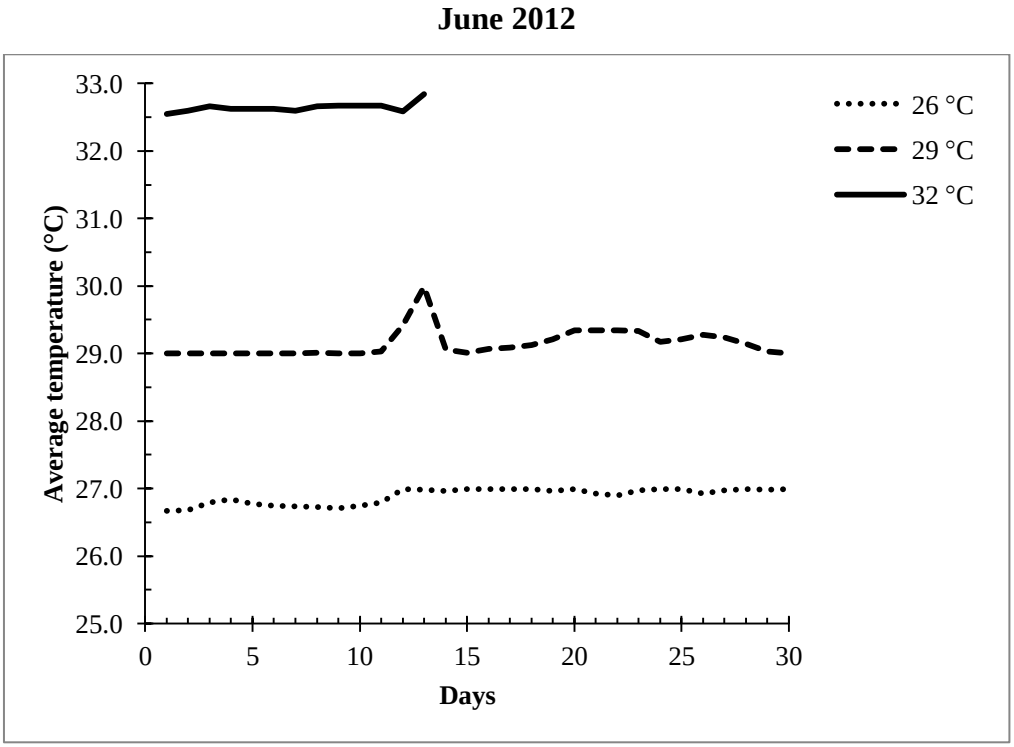

Figure B13 Average daily temperature in microprocessor-controlled incubators at different incubating temperatures in June 2012.

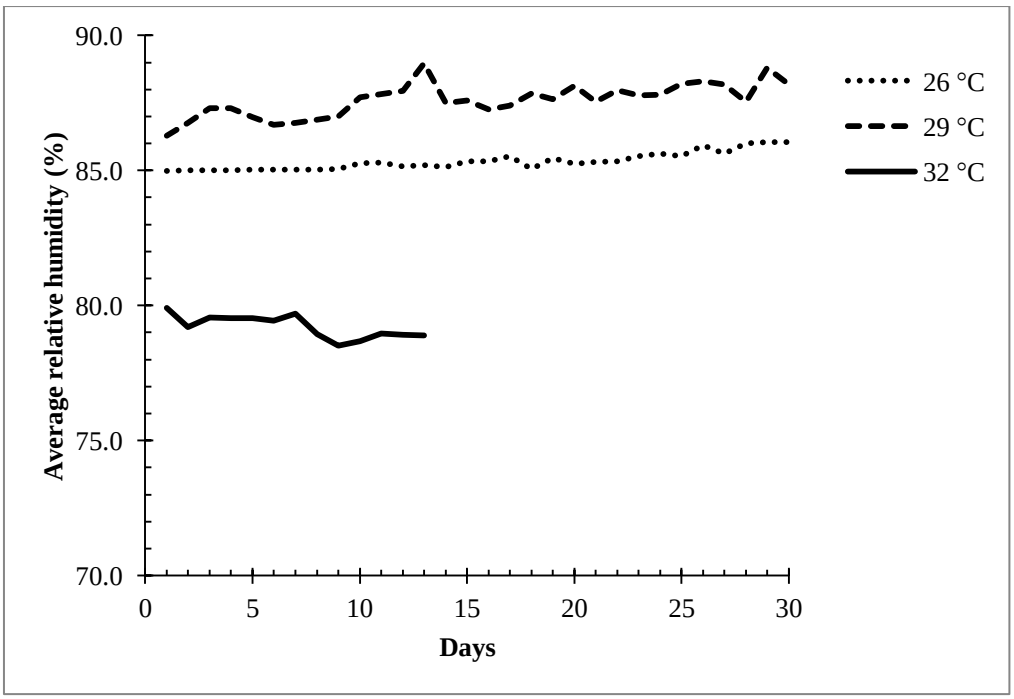

Figure B14 Average daily relative humidity in microprocessor-controlled incubators at different incubating temperatures in June 2012.

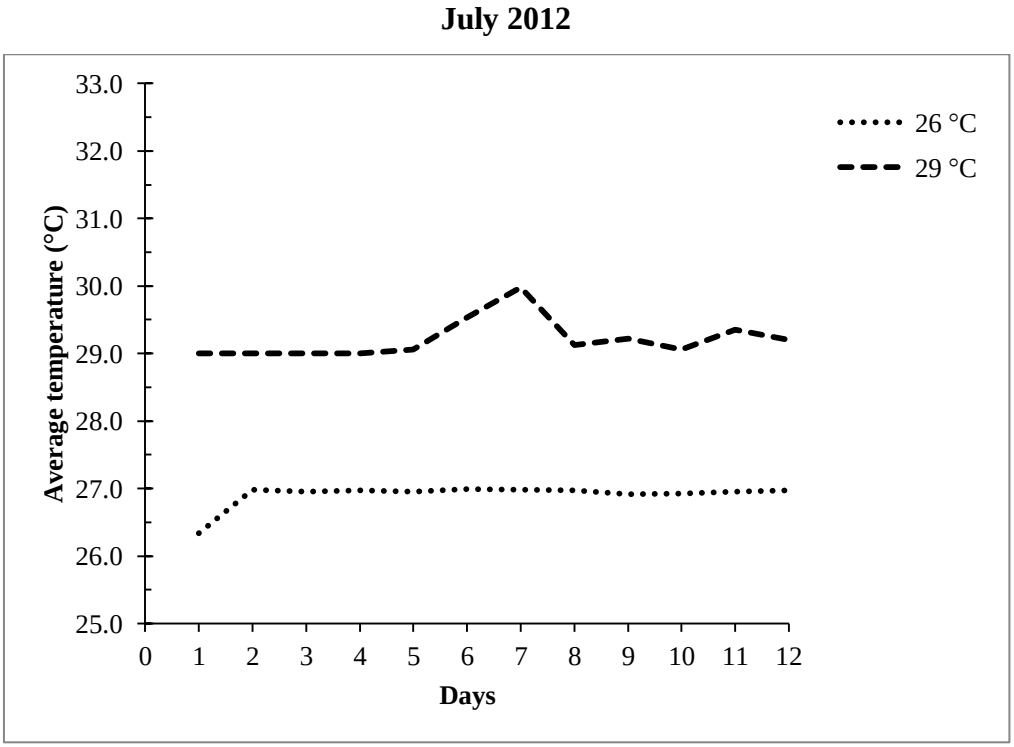

Figure B15 Average daily temperature in microprocessor-controlled incubators at different incubating temperatures in July 2012.

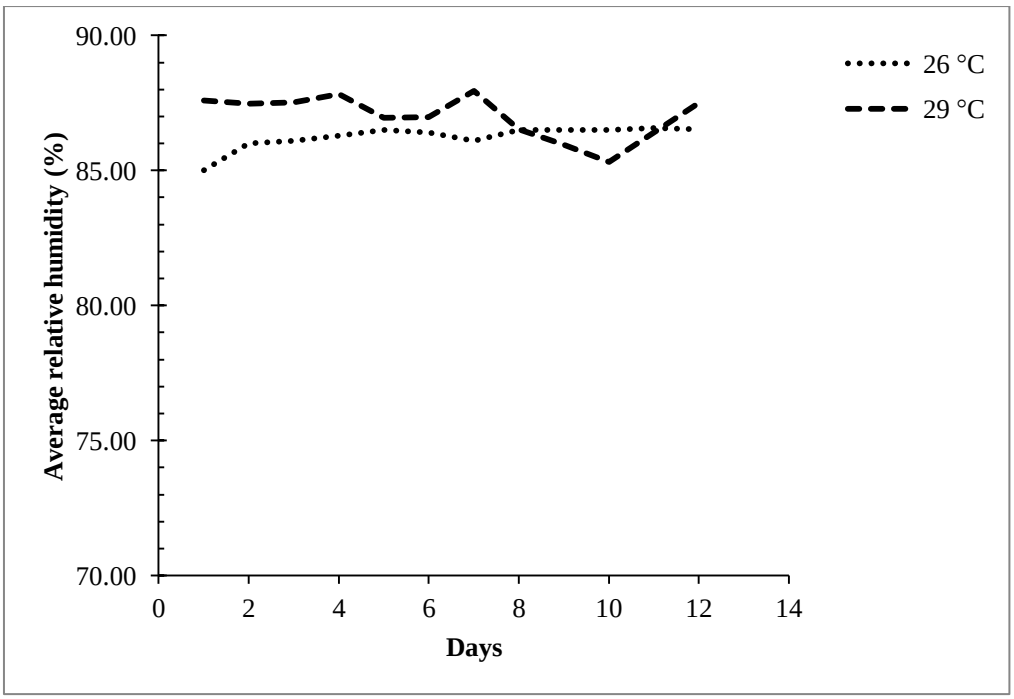

Figure B16 Average daily relative humidity in microprocessor-controlled incubators at different incubating temperatures in July 2012.

BIOGRAPHY

Miss Rangsima Pewphong was born on the 24th of April 1988 in Bangkok, Thailand. She graduated a Bachelor of Science in Biology from the Department of Biology, Faculty of Science, Srinakharinwirot University since 2010. After her graduation, she continued her graduate study at the Department of Biology, Faculty of Science, Chulalongkorn University for Master of Science in Zoology with a full financial support from the Human Resource Development in Science Project (Science Achievement Scholarship of Thailand, SAST) since 2010. During her graduate study, she has presented parts of her work in form of oral and poster presentations in the national and international conferences including the 17th Biological Sciences Graduate Congress at Chulalongkorn University in December 2012 (poster presentation), the 8th Conference on Science and Technology for Youths at Bangkok International Trade & Exhibition Centre in March 2013 (oral presentation) and the International Conference on Environmental and Hazardous Substance Management at Imperial Queen's Park Hotel, Bangkok in May 2013. She has already published a part of her work as a research article in the Proceedings of the 8th Conference on Science and Technology for Youths.