Tropical Natural History 21(3): 354-364, December 2021

Nesting Ecology and Reproducing Females of the Snail-eating Turtle, *Malayemys macrocephala,* in Paddy Fields in Central Plain of Thailand

YUPAPORN VISOOT[1], THONGCHAI NGAMPRASERTWONG[1,2] AND NOPPADON KITANA[1,2*]

[1]*Program in Zoology, Department of Biology, Faculty of Science, Chulalongkorn University, 254 Phayathai Road, Pathumwan, Bangkok, 10330, THAILAND*
[2]*BioSentinel Research Group (Special Task Force for Activating Research), Department of Biology, Faculty of Science, Chulalongkorn University, 254 Phayathai Road, Pathumwan, Bangkok 10330, THAILAND*
[*]Corresponding author. Noppadon Kitana (noppadon.k@chula.ac.th)
Received: 5 May 2021; Accepted: 25 November 2021

ABSTRACT.– The Malayan snail-eating turtle, *Malayemys macrocephala*, is a common freshwater turtle that is distributed in the central plain of Thailand. The turtle is well adapted to agricultural areas, making them vulnerable to population decline as their nesting grounds are not in protected areas. This study aimed to examine the nesting ecology and estimated number of reproductive females. Nests of *M. macrocephala* were surveyed in a 16-hectare rice field at central Thailand. Turtle nests were searched for and monitored on two consecutive days per fortnight during December 2013 to May 2014. The nesting season of *M. macrocephala* in this area was from early December to early April. A total of 74 clutches of 3–9 eggs each were found in this area. Turtle nests were covered by ground vegetation and mainly found on the rice-field ridge with the distance less than 5 m from the water source. All the turtle eggs were collected for incubation at the laboratory until hatching. In addition, adult turtles were trapped by mesh traps placed in canals within the study area. Mitochondrial DNA samples of juvenile turtles and trapped adult females were analyzed for haplotype variation in the *cytochrome b* gene. From 71 clutches and six adult females found within this study period, seven haplotypes were found. This indicates that seven or more females reproduced. Given the assumption that a reproductive female lays only one clutch per night, eggs with the same haplotype in multiple new nests in one night were laid by different females. Based on this, our field records of newly formed nests revealed that at least 10 different females reproduced in this 16-hectare area. Our results show that combining field and molecular techniques allows for determining a minimum number of female turtles that reproduce in a particular area, which is useful for conservation planning in the future.

KEY WORDS: *cytochrome b*, freshwater turtle, mitochondrial DNA, nesting-site preference, reproducing female number

INTRODUCTION

Snail-eating turtles belong to the genus *Malayemys* and are classified into the three species of *M. subtrijuga*, distributed in the Mekong River Basin, *M. macrocephala*, in the Chao Phraya and Mae Klong River Basins and the coastal area of south-eastern Thailand, and *M. khoratensis*, in the Khorat plateau (Brophy, 2004; Ihlow et al., 2016). Turtles previously classified as *M. subtrijuga* in the central part of Thailand and adjacent areas may have been *M. macrocephala* (Brophy, 2004). Regardless of the species, snail-eating turtles have been exploited for decades, where their meat and eggs serve as an important protein source and the shell as traditional medicine. In Thailand, snail-eating turtles have frequently been trapped and relocated for religious-related activities. *Malayemys macrocephala* is protected by the Wild and Protected Animal Act of Thailand (1992 and 2015) and the Convention on International Trade in Endangered (CITES) Appendix II. However, its habitats in agricultural areas are not protected by the law, leaving them vulnerable to exploitation as well as susceptible to

agrochemical use (Keithmaleesatti, 2008). Nonetheless, only few studies provide dependable knowledge necessary for efficient conservation management of snail-eating turtles.

With the lack of parental care, snail-eating turtles may be subjected to strong selection on nest-site choice by females for offspring survival (Escalona et al., 2009). The environment in the nesting site influences incubation duration, hatching success and sex of the embryo. Physical factors, such as the nest depth, distance from water, temperature, humidity and shading level, also affect the hatching success (Congdon et al., 1983; Kipp, 2003; Ferreira and Castro, 2010). Considering that the suitable nesting environments may depend on species, it is important to investigate specific nesting ecology for sustainable conservation of snail eating turtles.

The number of reproductive individuals plays an important role in maintaining a sustainable population size. There may need to be 50 reproductive individuals at minimum for a population to avoid inbreeding and extinction (Franklin, 1980). The number of reproductive individual turtles was studied by direct observation, but this is difficult and time consuming, especially in nocturnal species (Congdon et al., 1983; Congdon and Gibbons, 1990; Congdon et al., 2000). The number of nests may represent the number of reproductive female turtles. However, this is invalid in many turtle species where single reproductive female can lay multiple clutches in a single nesting season (Lebuff and Beatty, 1971). Recently, molecular analysis provided an indirect approach to study reproductive strategies that are difficult to observed directly (Pearse et al., 2002).

In this research, the nesting ecology of the snail-eating turtle *M. macrocephala* in paddy fields at a site in central Thailand was studied to elucidate nest site preference. Furthermore, nucleotide sequences in a partial mitochondrial DNA fragment of the *cytochrome b (Cytb)* gene from hatchlings from nests as well as trapped adult females were used to estimate the number of reproducing females and predict multiple nesting strategies of *M. macrocephala*. Such information on the nesting ecology and number of reproductive females is crucially important for drafting appropriate conservation measures for this turtle.

MATERIALS AND METHODS

Animal procedures, including egg collection, turtle trapping, turtle husbandry and blood sampling, were conducted in accordance with the Wild and Protected Animal Act of Thailand (1992) and had been approved by the Department of Fisheries, Ministry of Agriculture and Cooperatives (Permit Number 18/2559) and the Chulalongkorn University Animal Care and Use Committee (Protocol Review Number 1623012)

Study Area.

The study area was located in a 16-hectare agricultural area of Sena District, Phra Nakhon Si Ayutthaya province (14.399875–14.395864° N and 100.390575–100.388902° E), where a large area of habitat including nesting sites of *M. macrocephala* has been recorded previously (Keithmaleesatti, 2008; Pewphong, 2012). The present paddy area was divided into arbitrary blocks by soil ridges (Fig. 1).

Nesting ecology.

Nests of *M. macrocephala* were searched for by visual encounter surveys during daytime (8:00 AM to 4:00 PM) for two consecutive days per fortnight from November 2013 to April 2014. This period of field surveys effectively covers the annual period when *M. macrocephala* reproduces in this area (Pewphong, 2012). Nest locations were recorded with a GPS navigation device and plotted by ArcGIS Online (WGS84 system). The distance from each nest found to the nearest water source was recorded on site.

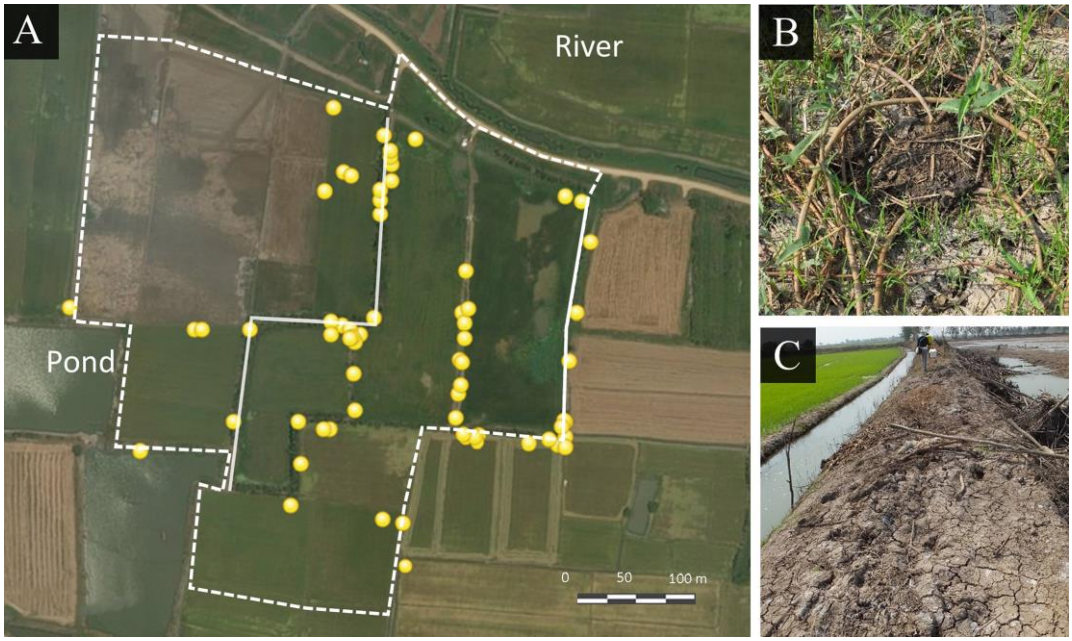

FIGURE 1. Study area in a 16-hectare agricultural area of Sena District, Phra Nakhon Si Ayutthaya province, central Thailand (14.399875–14.395864 °N and 100.390575–100.388902 °E). *Malayemys macrocephala*'s nest locations were marked as the yellow dots. (A) The character of study area, where the dotted line represents the boundary of the study area, and the white lines represent the canals where mesh traps were placed. (B) *M. macrocephala*'s nest and (C) rice-field ridge with nearby irrigation canal.

Right after the nest's covering materials was opened, temperatures at the uppermost portion of the nest and at the eggshell surface were recorded with an infrared thermometer (Raytek Minitemp MT4). Nest size was measured in depth, width and height. Soil sample from inside the nest was collected and kept in a zip-lock bag for measuring pH by digital pH meter (Hanna Instrument Checker® HI98103) and soil humidity. The plant type and area percentage of plant cover in a 30 x 30 cm quadrat above the nest position was recorded as vegetation cover.

Egg incubation.

Clutch size in each nest was recorded and all the eggs were then collected and labeled individually and placed in Styrofoam box before transporting to a laboratory at Chulalongkorn University. After cleaned with water, each egg's weight and size were measured. They were placed in plastic boxes containing moist vermiculite (1 g vermiculite/ 1 mL distilled water) and incubated in a microprocessor controlled incubator at 29 °C. The relative humidity in the incubator was maintained above 80% by placing trays of water inside the incubator (Keithmaleesatti, 2008). Upon hatching, each hatchling was weighed and kept in the incubator for additional 2 weeks to allow for complete closure of its plastron. Hatchlings were raised individually in a 12 cm width x 20 cm length x 9 cm height plastic aquarium containing 700 mL de-chlorinated tap water and a clay platform. Hatchlings were kept under semi-natural condition and fed with commercial pellet until blood sampling.

Turtle trapping.

During the same nesting season, turtles were collected from the study site by mesh

traps. The traps were placed in the water body nearby the nesting area (Fig. 1) in the evening (06:00 PM) and retrieved 12 h afterwards at 06:00 AM. To reduce interference with nesting, trapping was performed every other week alternating with the egg collection week. Trapped turtles were weighed, size- measured and sexed. Adult turtles were classified as having a maximum plastron length of > 100 mm (Brophy, 2004). All turtles were marked on their shell with a plastic Dymo® tag and silicone glue before released back at the trapping spot.

Blood sampling and mitochondrial DNA analysis.

Blood samples of adult female and juvenile turtles were taken from the subcarapacial sinus using a 25-gauge and 27-gauge needle, respectively, (Heard et al., 2004). Total DNA was extracted from each blood sample using 5 PRIME ArchivePure DNA Cell/Tissue Kit and BIOFACT™ Genomic DNA Prep Kit. Mitochondrial *Cytb* DNA was PCR amplified with the universal primer pair L14724 5'-CGAAGCTTGATATGAAAAACCATCGTTG-3' and H15149 5'-AACTGCAGCCCCTCAGAATGATATTTGTCCTCA-3' (Irwin et al., 1991). The PCR amplifications were performed in a reaction mixture of 10 µL containing 2 µL of genomic DNA (50 ng/µL), 0.5 µL of each primer (10 mM) and 7 µL of i-Taq™ Plus DNA polymerase. The amplifications were conducted in a thermal cycler (MJ Research Thermal Cycler Dual 48) with the following conditions: 95 °C for 5 min then 35 cycles of 94 °C for 45 s, 50 °C for 45 s and 72 °C for 1 min followed by 72 °C for 5 min, 20 °C for 1 min and 4 °C for 10 min. Thereafter the PCR products were visualized and checked for expected size (ca. 410 bp) under transillumination after coresolution with a DNA ladder (SibEnzyme® 100bp + 1.5 Kb) through 1% (w/v) agarose gel- electrophoresis (UltraPure™ Agarose) and staining with RedSafe™ Nucleic Acid Staining Solution (20,000x). The PCR amplicons were then purified with FavorPrep GEL/PCR purification mini kit before commercial sequencing with an ABI 3730XL DNA Analyzer at BiONEER (Republic of Korea) using L14724. The obtained sequences were verified by comparison with other annotated sequences available in the GenBank database using the BLASTn algorithm, and aligned by Muscle in the MEGA 7 program.

Statistical analysis

Nesting variables (e.g., soil moisture) were tested for normality and equality of variance using the Kolmogorov Smirnov and Levene test prior to mean comparison by an independent samples t-test. Chi-square tests were used to compare proportion data (e.g., proportion of nest with different distance from the nearest water source and vegetation cover). Correlation between measured variables was analyzed by Spearman's correlation test. The statistical tests were performed on IBM SPSS statistics version 22.0.

RESULTS

Nesting ecology.

Within the 16-hectare study area in central Thailand, the first nest of *M. macrocephala* was found in early December 2013 and the last one was found in early April 2014. A total of 74 nests were found in the study area, with the highest number of nests being found around late February to early March, accounting for 37.8% of all nests (Table 1).

Nests was located 5.3 ± 1.74 cm (mean ± SD) below the soil surface. Nest chambers were 7.3 ± 1.66 cm wide and 8.7 ± 1.41 cm deep. Temperatures were 25.70 ± 3.40 °C in nests and 25.47 ± 3.17 °C on the egg surface. Nest soil moisture varied in the range of 7.28–69.81% depending on the time of nest building, where nest built the night before collecting day or freshly built nests showed a significantly higher soil moisture than the

TABLE 1. Number of *Malayemys macrocephala* nests found at the 16-hectare agricultural area in central Thailand on the first and second survey days of each fortnight during the nesting season (December 2013 to April 2014)

	Early Dec.	Late Dec.	Early Jan.	Late Jan.	Early Feb.	Late Feb.	Early Mar.	Late Mar.	Early Apr.
1st day	2	2	3	10	9	12	10	6	3
2nd day	0	1	4	3	1	2	4	1	1
Total	2	3	7	13	10	14	14	7	4

older ones (mean 32.57% and 22.56% respectively; $t = 3.24$; $df = 53.07$; $p = 0.002$). The soil inside the turtle's nest was usually acidic with a pH in the range of 3.01–7.22.

Two types of water source were found in the paddy field in the study area. These were relatively stable water sources, such as canals and ponds, and temporary water sources, such as water in the cultivated area. The distance from each nest to the nearest water source was evaluated in five categories of 5-m increments. Fifty-two nests (70%) were found within 5 m, 11 nests (15%) at 6–10 m, 4 nests (6%) at 11–15 m, 6 nests (8%) at 16–20 m and only 1 nest (1%) more than 20 m from the water source. The proportion of nests within 5 m of the water source was significantly higher than that of the rest found in more distant places ($\chi^2 = 117.07$; $p < 0.001$).

Among the 74 nests found in this study, 64 nests (86%) were covered with some form of vegetation or plant debris. Thirty-four nests were covered by vegetation, such as herbs, grasses and vines, twenty-eight by debris, and two by both vegetation and debris. The percentage of vegetation cover on the nest, in terms of the surface area covered, varied. No significant difference in the degree of vegetation was found among these nests ($\chi^2 = 6.04$; $p = 0.302$). There was no significant correlation between percentage of vegetation cover and nest temperature in this species (Spearman's rho = 0.082; $p = 0.492$).

Clutch size and hatching success.

On average (mean ± SD), the clutch size of *M. macrocephala* found in this study was 6 ± 1.11 with a range of 3–9 eggs. The egg was elongate with 22.92 ± 3.02 mm width and 38.87 ± 3.60 mm length. Egg weight was 12.20 ± 1.72 g. Upon laboratory incubation, 293 eggs hatched from 423 eggs collected (69.3% hatching success). The earliest hatch occurred in early April 2014 and the last hatch occurred in late November 2014, with the peak of hatching in July 2014. The majority of eggs hatched in July 2014 were collected during the peak of nesting activity (February 2014, Table 2). It is interesting to note that incubation period of the snail-eating turtle was vary from 82-250 days (with an average 153 ± 37 days) under laboratory condition and eggs from the same clutch did not hatch at the same time. The difference in time between the first and the last hatching was 51 ± 34.6 day apart (range of 3–121 days).

Trapping success.

The total number of trapped *M. macrocephala* during December 2013 to April 2014 was 17 individuals, comprised of seven different males (two adults and five juveniles) and 10 females (six adults and four juveniles) (Table 3). The highest number of trapped male in a month was three in January, while only one male was trapped in each of February and March. The number of female turtles caught in January, February and March were 2, 6 and

TABLE 2. Hatching of *Malayemys macrocephala* eggs after incubation in the laboratory at (29 °C, ≥ 80% relative humidity). Number of hatched eggs are sorted according to egg collecting month and hatching month.

Hatching month	Egg collecting month					
	Dec.	Jan.	Feb.	Mar.	Apr.	Total
Apr.	2	13	2	-	-	17
May.	17	21	17	3	-	58
Jun.	4	15	15	10	-	44
Jul.	3	31	56	39	1	130
Aug.	-	3	3	3	2	11
Sep.	-	1	1	11	-	13
Oct.	-	-	1	7	4	12
Nov.	-	-	-	5	3	8
Total	26	84	95	78	10	293

3 individuals, respectively. It is of interest to note that female turtle number F14 and F18 were captured in February and re-captured again in March. However, no individual was caught in April 2014. The number of trapped females seemed to relate to the number of nests found in each month.

Mitochondrial DNA analysis.

Among the 74 nests found in this study, 71 nests were successfully analyzed for mitochondrial DNA variation. Missing nests data were due to the unfertilized eggs and the death in embryogenesis. Sequence alignment of the 412 bp fragments of *Cytb* revealed seven different haplotypes. These haplotypes were confirmed to be *M. macrocephala* with 99% DNA sequence similarity to sequences annotated as *M. macrocephala* in the GenBank database.

These seven haplotypes were nominally categorized as haplotypes A to G with GenBank accession numbers MW027838-MW027844. Among these haplotypes, twelve variable sites resulting from 2 transition and 10 transversion mutations. The number of nests of each haplotype was 24, 15, 5, 22, 3, 1 and 1 nest(s) for haplotypes A–G, respectively (Fig. 2). Haplotypes A, B, C and D were found regularly during the nesting season, while haplotypes E, F and G were only found in February 2014, December 2013 and January 2014, respectively. Among the trapped six adult females, F09, F12 and F14 had haplotype A, and F15, F16 and F20 each had haplotypes B, C and D (Table 3).

DISCUSSION

Nesting ecology.

In the previous study in this region, the nesting season of *M. macrocephala* was from November until April (Keithmaleesatti, 2008). In this study period (2013–2014), however,

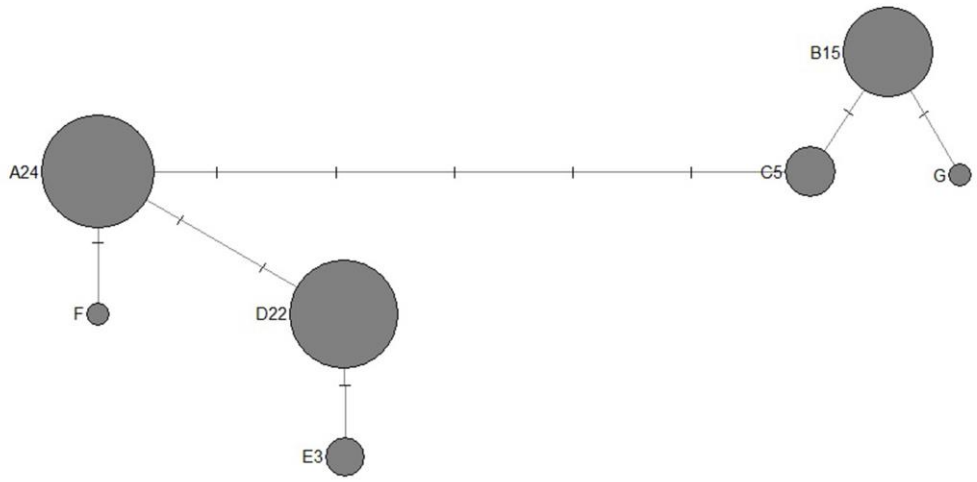

FIGURE 2. Haplotype network of 7 different Cytb haplotypes (412 bp) of *Malayemys macrocephala* from agricultural area at Sena district, Phra Nakhon Si Ayutthaya province. Circle size is proportional to number of samples in each haplotype.

the paddy field in Phra Nakhon Si Ayutthaya province was flooded until November. Therefore, the first nest was found in early December. This is similar to the report by Pewphong et al. (2013) that the onset of the nesting season in *M. macrocephala* was delayed for almost a month due to the lack of above water-level nesting ground sites during the 2011 flood.

For the visual encounter survey of turtle nests, a new nest can be identified by the moist surface of the covering materials. Indeed, new nests were found to have a significantly higher soil moisture than the older ones. This could be due to the fact that female turtles use water kept in their bursae near the cloaca to soften the soil before digging for oviposition (Smith and James, 1958). It is also important to note that *M. macrocephala*'s nests were found only on the rice-field ridge, even when there was no cultivar in the paddy field. The nesting site pattern of turtles could be ascribed to either adaptive nest-site selection or social facilitation (Escalona et al., 2009). Under a social facilitation nesting pattern, the nesting site is unpredictable, and nests are often clustered, as found in *Podocnemis unifili* (Escalona et al., 2009). However, the nesting pattern of *M. macrocephala* seems to fit more with an adaptive nest-site selection pattern. The nests were only located on the ridge (non-random site) mostly in a limited range of distance from water source under vegetation covering the ground.

The distance between the turtle's nest to the water source can affect the nesting success. Nests built close to water may be at risk from flooding and predation of the eggs and embryos (Marchand et al., 2002; Morjan, 2003). On the other hand, nests far from water have an increased risk of desiccation, hatchling disorientation, soil erosion and predation of egg-laying mothers and hatchlings after hatching (Congdon et al., 2000; Steen et al., 2006). This study shows that most of the gravid female *M. macrocephala* laid their eggs quite close to the water source (70% of nests laid within 5 m), presumably for a high humidity in the

TABLE 3. *Malayemys macrocephala* caught in the 16-hectare agricultural area in central Thailand during the 2013–2014 nesting season (Remark: PL = plastron)

Trapped date	Label	Sex	Weight (g)	PL Width (mm)	PL Length (mm)	Maturity
5 Dec. 2013	M01	Male	110	40	75	Juvenile
18 Dec. 2013	M03	Male	156	80	83	Juvenile
2 Jan. 2014	M06	Male	94	60	70	Juvenile
	M07	Male	980	129	155	Adult
14 Jan. 2014	F09	Female	260	88	100	Adult
	F10	Female	135	71	80	Juvenile
	M11	Male	270	90	105	Adult
2 Feb. 2014	F12	Female	1,110	142	164	Adult
	F13	Female	165	80	89	Juvenile
13 Feb. 2014	F14	Female	1,325	153	182	Adult
	F15	Female	943	133	160	Adult
	F16	Female	478	104	127	Adult
	F18	Female	48	53	53	Juvenile
26 Feb. 2014	M19	Male	187	72	92	Juvenile
	F18	Previously trapped in 13 Feb. 2014				
12 Mar. 2014	F20	Female	1100	154	170	Adult
	F21	Female	88.93	65	68	Juvenile
26 Mar. 2014	M22	Male	120.49	67	83	Juvenile
	F14	Previously trapped in 13 Feb. 2014				

nest, since it was reported that under laboratory conditions a relative humidity above 80% was needed for optimal *M. macrocephala* hatching success (Keithmaleesatti, 2008).

In numerous freshwater turtles, clean sandy substrates with no or minimal vegetation cover are used as nesting ground (Ernst and Barbour, 1989; Ernst et. al., 1994; Pritchard, 1979; Pritchard and Trebbau, 1984; Lindeman, 2001). However, 86% of *M. macrocephala* nests was found under high percentage of vegetation cover. This could be explained by the fact that the covered nests were usually found in habitats that are thinner shaded and less moist than the other sites (Congdon and Gibbons, 1990). Although,

nests surrounded by more vegetation cover are better buffered from environmental temperature shifts (Weisrock and Janzen, 1999), no direct association between vegetation cover and nest temperature was found in this species. Vegetation cover may play an important role in reducing nest predation. Turtle's eggs predators can detect turtle's nest by visuality (soil surface disturbance) and scent (the odor that nesting female left over) (Strickland et. al., 2010). In sea turtle's nests laid on the coasts of Baja California, a lower predation rate was found when the nesting site was near or among vegetation (Méndez-Rodríguez and Álvarez-Castañeda, 2016). In addition to humans, the main predators of *M. macrocephala's* eggs and offspring are olfactory predators, such as rodents and water monitor lizards (Keithmaleesatti, 2008). Female turtles may attempt to use ground vegetation and debris to camouflage (reducing level of soil surface disturbance) and reduce the nest's odor. However, nests that are covered by living herbaceous plants can be intruded by growing roots (Congdon et al., 2000). The plant's roots may tighten around the turtle's eggs and can make the nest's covering materials too hard to dig out from. In this study, most nests were covered by living plants that could grow during the egg incubation period (around 3 months), and so the negative effect of nesting under vegetation in this species is still possible and needs further study.

Non-synchronous hatching.

Synchronous hatching is commonly found in marine turtles and some freshwater turtles, such as *Chrysemys picta* and *Emydura macquarii* (De Pari, 1996; Spencer et al., 2001). Synchronous hatching could dilute the predation risk by reducing individual's exposure to prey-switching predators (Tucker et al., 2007; Colbert et al., 2010). Synchronous hatching may be induced by embryos or hatchling activities, including movement, vibration and sound (Spencer et al., 2001; Colbert et al., 2010) as well as by abiotic environmental cues (Doody, 2011). In the present results, broods of *M. macrocephala* did not exhibit synchronous hatching within clutches in the controlled temperature and humidity regime inside an incubator. These eggs incubated in the same plastic box did not hatch within short periods expected in synchronous hatching. This rules out that hatchling activities trigger other siblings' hatching in this species. It is of importance to note that some freshwater turtles rely heavily on environment cues for synchronous hatching. *Carettochelys insculpta* can delay hatching until the nest is inundated in the wet season (Webb et al., 1986). *Chelonia rugosa*'s hatchling may not emerge for many weeks until heavy rains soften the dried mud and flood the nest (Kennett et.al., 1993a, b). The present case of asynchronous hatching in *M. macrocephala* might have resulted from the absence of environmental cues known as a trigger for synchronous hatching in a freshwater turtle. In the present laboratory condition, most of the eggs hatched in the period of wet season. Thus, in *M. macrocephala,* an abiotic cue such as heavy rains may trigger synchronous hatching in their natural habitats.

Multiple nesting and estimated number of reproducing females.

In this study, mitochondrial *Cytb* DNA analysis detected seven haplotypes in six adult females and 71 clutches of *M. macrocephala*. Therefore, at least seven females nested in the study area. There has been no available evidence that sexually mature females produce more than one clutch of eggs in one night. Although *Batagur baska* occasionally divide a clutch of eggs into several clutches in one night (Moll, 1980), there has been no available evidence that sexually mature females of a smaller turtle produce more than one clutch of eggs in one night. Thus, when identical haplotypes were found from eggs among multiple nests that were formed in the

same night, those nests had most probably been made by different females. Based on this assumption, two different females would have laid eggs with haplotype B in two new nests found in the same night in March. Similarly, eggs with haplotype D in three nests, which were newly found on one night in January, would have been made by three females. These cases add three to the seven. Therefore, our results provide strong empirical support that at least 10 females reproduced in the study area in this period. The number of nests that were newly found in the same nights are likely to be built by different females. Field records of multiple nests formed in different nights are, however, insufficient to know how many different females could have reproduced over the period. On the other hand, eggs with different mtDNA haplotypes can only be produced by different females regardless of when they were laid. Our study exemplified taking advantage of these merits of field and mtDNA haplotype data to determine the minimum number of female turtles that reproduced in a given area and period.

CONCLUSION

The nesting ecology of *M. macrocephala* suggested an adaptive nest-site selection strategy. Gravid females of *M. macrocephala* lay eggs in nests made on the rice-field ridge close to the surface water and cover the nests with vegetation and debris. This study presents quantitative information of nesting ecology that could be used for turtle protection and sustainable management by predicting nest sites for protection. Based on field and molecular techniques, at least 10 reproducing females with seven different haplotypes used this 16-hectare area as a nesting ground.

ACKNOWLEDGEMENTS

This research was supported by the 90th Anniversary of Chulalongkorn University Fund (Ratchadaphiseksomphot Endowment Fund) and the Department of Biology, Faculty of Science, Chulalongkorn University. Thanks to Mr. Wichian Korpradap, the owner of the study site, for allowing us to survey for nest and eggs as well as to trap turtles in this area, and Dr. Robert D. J. Butcher of the Research Clinic, Chulalongkorn University for editing the English language of this manuscript.

LITERATURE CITED

Brophy, T.R. 2004. The South-East Asian turtles of the genus *Malayemys* (Testudines: Bataguridae). Hamadryad, 29: 63-79.

Colbert, P.L., Spencer, R.J. and Janzen, F.J. 2010. Mechanism and cost of synchronous hatching. Functional Ecology, 24(1): 112-121.

Congdon, J.D. and Gibbons, J.W. 1990. Turtle eggs: Their ecology and evolution. In: Gibbons, J.W. (Ed.). Life History and Ecology of the Slider Turtle, Smithsonian Institution Press, Washington D.C., 109-123.

Congdon, J.D., Nagle, R.D., Kinney, O.M., Osenioski, M., Avery, H.W., van Loben Sel, R.C. and Tinkle, D.W. 2000. Nesting ecology and embryo mortality: Implications for hatching success and demography of Blanding's turtles (*Emydoidea blandingii*). Chelonian Conservation and Biology, 3(4): 569-579.

Congdon, J.D., Tinkle, D.W., Breitenbach, G.L. and van Loben Sel, R.C. 1983. Nesting ecology and hatching success in the turtle *Emydoidea blandingi*. Herpetologica, 39(4): 417-429.

De Pari, J.A. 1996. Overwintering in the nest chamber by hatchling painted turtles, *Chrysemys picta*, in northern New Jersey. Chelonian Conservation and Biology, 2: 5-12.

Doody, J.S. 2011. Environmentally cued hatching in reptiles. Integrative and Comparative Biology, 51(1): 49-61.

Escalona, T., Valenzuela, N. and Adams, D.C. 2009. Nesting ecology in the freshwater turtle *Podocnemis unifilis*: Spatiotemporal patterns and inferred explanations. Functional Ecology, 23(4): 826-835.

Ernst, C.H., Barbour, R.W. 1989. Turtles of the World. Smithsonian Institution Press, Washington D.C., 313 pp.

Ernst, C.H., Lovich, J.E. and Barbour, R.W. 1994. Turtles of the United States and Canada. Smithsonian Institution Press, Washington D.C., 578 pp.

Ferreira, P.D. and Castro, P.T.A. 2010. Nesting ecology of *Podocnemis expansa* (Schweigger, 1812) and *Podocnemis unifilis* (Troschel, 1848) (Testudines, Podocnemididae) in the Javaés River, Brazil. Brazilian Journal of Biology, 70(1): 85-94.

Franklin, I. R. 1980. Evolutionary Change in Small Populations. Sinauer, Sunderland, MA. 135-149 pp.

Heard, D.J., Harr, K.E. and Wellehan, J. 2004. Diagnostic Sampling and Laboratory Tests. BSAVA, Gloucester.

Ihlow, F., Vamberger, M., Flecks, M., Hartmann, T., Cota, M., Makchai, S., Meewattana, P., Dawson, J. E., Kheng, L., Rödder, D. and Fritz, U. 2016. Integrative taxonomy of Southeast Asian snail-eatingt turtles (Geoemydidae: *Malayemys*) reveals a new species and mitochondrial introgression. PLoS ONE. 11(4): doi:10.1371/journal.pone.0153108.

Irwin, D.M., Kocher, T.D. and Wilson, A.C. 1991. Evolution of the *Cytochrome b* gene of mammals. Journal of Molecular Evolution, 32: 128-144.

Keithmaleesatti, S. 2008. An Assessment of the Association of Organochlorine Pesticide Contamination and Reproductive Effects on the Snail-eating Turtle *(Malayemys macrocephala*) in the Lower Chao Phraya River Basin, Thailand. Doctoral Dissertation, Chulalongkorn University, Bangkok, Thailand, 143 pp.

Kennett, R., Christian, K. and Pritchard D. 1993a. Underwater nesting by the tropical freshwater turtle, *Chelonia rugosa* (Testudinata: Chelidae). Australian Journal of Zoology, 41: 47-52.

Kennett, R., Georges, A. and Plamer-Allen M. 1993b. Early developmental arrest during immersion of eggs of a tropical freshwater turtle, *Chelonia rugosa* (Testudinata: Chelidae) from northern Australia. Australian Journal of Zoology, 41: 37-45.

Kipp, R.L. 2003. Nesting Ecology of the Eastern Box Turtle (*Terrapene carolina carolina*) in a Fragmented Landscape. Master's Thesis, University of Delaware, USA, 78 pp.

Lebuff, C.R. and Beatty, R.W. 1971. Some aspects of nesting of the loggerhead turtle, *Caretta caretta caretta* (Linne), on the gulf coast of Florida. Herpetologica, 27(2): 153-156.

Lindeman, P.V. 2001. Notes on nesting by the smooth softshell turtle (*Apalone mutica*) in a river impoundment in Western Kentucky. Journal of the Kentucky Academy of Science, 62: 117-120.

Marchand, M.N., Litvaitis, J.A., Maier, T.J. and DeGraaf, R.M. 2002. Use of artificial nests to investigate predation on freshwater turtle nests. Wildlife Society Bulletin, 30(4): 1092-1098.

Méndez-Rodríguez, L. and Álvarez-Castañeda, S.T. 2016. Predation on turtle nests in the southwestern coast of the Baja California Peninsula. Revista Mexicana de Biodiversidad, 87(2): 483-488.

Moll, E.O. 1980. Natural history of the river terrapin, *Batagur baska* (Gray) in Malaysia (Testudines: Emydidae). Malaysian Journal of Science, 6: 23-62.

Morjan, C.L. 2003. Variation in nesting patterns affecting nest temperatures in two populations of painted turtles (*Chrysemys picta*) with temperature-dependent sex determination. Behavioral Ecology and Sociobiology, 53(4): 254-261.

Pearse, D., Janzen, F. and Avise, J. 2002. Multiple paternity, sperm storage, and reproductive success of female and male painted turtles (*Chrysemys picta*) in nature. Behavioral Ecology and Sociobiology, 51(2): 164-171.

Pewphong, R. 2012. Effects of Temperature on Gonadal Development of the Snail-eating Turtle *Malayemys macrocephala*. Master's thesis, Chulalongkorn University, Bangkok, Thailand, 129 pp.

Pritchard, P.C.H. 1979. Encyclopedia of Turtles. T.F.H. Publications, Neptune, NJ. 895 pp.

Pritchard, P.C.H. and Trebbau, P. 1984. The Turtles of Venezuela. Society for the Study of Amphibians and Reptiles. Ithaca, NY. 403 pp.

Smith, H.M. and James, L.F. 1958. The taxonomic significance of cloacal bursae in turtles. Transaction of the Kansas Academy of Science, 61(1): 86-96.

Spencer, R.J., Thompson, M.B. and Banks, P.B. 2001. Hatch or wait? A dilemma in reptilian incubation. Oikos, 29(3): 401-406.

Steen, D.A., Aresco, M.J., Beilke, S.G., Compton, B.W., Condon, E.P., Kenneth Dodd, C., Forrester, H.,. Gibbons, J.W, Greene, J.L., Johnson, G., Langen, T.A., Oldham, M.J., Oxier, D.N., Saumure, R.A., Schueler, F.W., Sleeman, J.M., Smith, L.L., Tucker, J.K. and Gibbs, J.P. 2006. Relative vulnerability of female turtles to road mortality. Animal Conservation, 9(3): 269-273.

Strickland, J., Colbert, P. and Janzen, F.J. 2010. Experimental analysis of effects of markers and habitat structure on predation of turtle nests. Journal of Herpetology, 44(3): 467-470.

Tucker, J.K., Paukstis, G.L. and Janzen, F.J. 2007. Does predator swamping promote synchronous emergence of turtle hatchlings among nests? Behavioral Ecology, 19(1): 35-40.

Webb, G.J.W., Choquenot, D. and Whitehead, P.J. 1986. Nests, eggs, and embryonic development of *Carettochelys insculpta* (Chelonia: Carettochelidae) from northern Australia. Journal of Zoology, 1: 521-550.