Tropical Natural History 10(2): 189-197, October 2010

Male Biased Sex Ratio in Captive Bred Siamese Narrow-headed Softshell Turtles, *Chitra chitra*: Does the Incubation Temperature Influence Hatchling Sex in the Family Trionychidae?

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Received: 11 June 2010; Accepted: 20 September 2010

ABSTRACT.– A captive breeding program for the critically endangered *Chitra chitra* (Testudines: Trionychidae) at Kanchanaburi Inland Fisheries Development Center (KIFDC), Thailand, produced 710 hatchlings between 2000 and 2004, with eggs being incubated in shaded areas only. Thirty-one captive bred *C. chitra* that died during rearing were dissected and their gonads examined histologically and microscopically to determined if they were ovaries or testes. Of the 29 samples that could be sexed, 23 were males and 6 were females (79% males). The male biased sex ratio in captive-bred *C. chitra* indicates that the incubation conditions at KIFDC, which are likely to be cooler than in natural nests on sun exposed river sand banks, may have had a masculinising effect on hatchlings. This is potentially contrary to the generally accepted wisdom that softshell turtles have genotypic sex determination and that incubation temperature has no effect on hatchling sex ratios. Although we cannot formally rule out differential developmental mortality between males and females, it is possible that sex determination in the family Trionychidae is more variable and complex than currently recognised. Our finding that the incubation environment in a captive breeding facility can produce skewed sex ratios in *C. chitra* has important implications for the recovery projects of other critically endangered softshell turtles.

KEY WORDS: Captive breeding, Incubation temperature, Environmental sex determination, Trionychidae

INTRODUCTION

While sex determination in most vertebrates depends on genotypic sex determination (GSD), where the zygote sex is determined by maternal and paternal genes and/or sex chromosomes, temperature -dependent sex determination (TSD) seems to be the most common form of sex determination in turtles. As a general rule for TSD in turtles, lower temperatures provide males and higher temperatures females (TSD Ia), although in some species very low temperatures can also provide females (TSD II) (Ewert et al., 2004). Exceptions to this rule include all species studied so far in the turtle families Trionychidae and Chelidae and the subfamily Staurotypinae within the Kinosternidae, as well as a few species of Emydidae and Bataguridae. These show GSD, with temperature having no influence on the offspring sex ratio (Ewert and Nelson, 1991; Janzen and Paukstis, 1991; Pieau, 1996; Kuchling, 1999; Janzen and Krenz, 2004; Moll and Moll, 2004; Sarre et al., 2004).

The sex determination mechanism of a given species is of practical importance in conservation programs involving captive breeding and/or the relocation of natural nests and/or artificial incubation, since under those scenarios TSD can lead to highly biased offspring sex ratios, whereas

GSD should provide an equal sex ratio independent of the incubation environment of the eggs. For this reason it is imperative to evaluate offspring sex ratios and / or the sex determination mechanism in all conservation programs for critically endangered turtles that involve manipulation of egg incubation.

The critically endangered (IUCN 2008) Siamese Narrow-headed Softshell Turtle *Chitra chitra* Nutphand, 1986, one of "the world's top 25 most endangered turtles" (Turtle conservation fund, 2003) is probably the largest and least known-about freshwater turtle, reaching a carapace length of up to 1.23 m (Kitimasak et al., 2005). Two subspecies have been described, the nominate form *C. chitra chitra* which occurs in Thailand and Malaysia and *C. chitra javanensis* from Indonesia (McCord and Pritchard, 2002). A captive breeding program for the Thai subspecies started in 2000 at Kanchanaburi Inland Fisheries Development Center (KIFDC), Kanchanaburi Province, Thailand (Kitimasak et al., 2003). On a certain level this project has had spectacular success: 983 eggs have been laid by two females between 2000 and 2004, 710 (72.2%) of which hatched. In the present paper we present data on the sex ratio of captive-bred juveniles that died during rearing to evaluate if the incubation methods used in the project provide a balanced sex ratio.

MATERIALS AND METHODS

Maintenance of breeding stock, egg handling, egg incubation methods and rearing of hatchlings and juveniles were described in detail by Kitimasak et al. (2003). In short, females nested on artificial sandbanks ascending 80 - 90 cm above the water level under roofed areas in ponds of 16 x 25 m (Fig. 1A). Nests were excavated by hand within 48 hours after nesting. Three methods were used for incubation: first, some clutches were incubated at the nesting areas *in situ* ("beach"). Second, eggs were incubated in circular fibreglass containers on 10 cm of sand, covered with 20 cm additional sand. The containers were placed in water under a roofed area at KIFDC ("drum"; Fig. 1B). Third, some eggs were put in plastic containers on top of 10 cm of sand and then covered with 20 cm of additional sand and maintained indoors at the Department of Biology, Chulalongkorn University, Bangkok. Max. – Min. mercury thermometers were inserted horizontally near the nests to monitor the incubation temperature throughout the incubation period. Clutch and egg parameters, incubation times, means and ranges of sand temperatures, hatching success, and the growth of hatchlings have been described by Kitimasak et al. (2003). Sand temperatures fluctuated between 24 and 39 °C, with most weekly means between 29 and 31 °C.

The gonads of 31 captive-bred juvenile *C. chitra* that had died during rearing were dissected and examined to differentiate between the testes and ovaries so as to sex the specimens. Seven of these specimens had been stored in a freezer and 24 had been preserved in 4% (w/v) formaldehyde (Table 1). Part of one gonad was removed and slightly squashed between glass slides for examination under a light microscope after staining with 0.01% (w/v) methylene blue in 1% (v/v) acetic acid aqueous solution for about 30 seconds. Moreover, parts of four formaldehyde preserved gonads were paraffin embedded according to standard histological techniques (Humason, 1979), sectioned (nominally 6 μm) and stained with Haematoxylin and Eosin for histological examination.

TABLE 1. Incubation, carapace length (CL) and sex (M = male, F = female) of dead juvenile *C. chitra*. "-" indicates that no information was available.

C. chitra ID	Preserv.[a]	Date of oviposition	Incubtion method	Days to hatching	Date of death	CL mm	Sex
259	Fo	04 Apr 2002	Beach	60	23 Jan. 2006	175	M
27	Fo	04 Apr 2002	Beach	60	27 Oct. 2003	183	M
260	Fr	04 Apr 2002	Beach	60	20 Jan. 2006	157	M
261	Fr	16 Mar – 20 Apr 2002	-	55 - 64	14 May 2005	141	M
262	Fr	18 Mar 2004	Drum	75	14 June 2005	92	M
263	Fr	22 Apr 2004	Drum	66 - 68	18 June 2005	95	M
1	Fo	28 Feb – 17 Mar 2003	Drum	53 - 79	01 Oct 2003	93	M
4	Fo	28 Feb – 17 Mar 2003	Drum	53 - 79	01 Oct 2003	87	M
17	Fo	28 Feb – 17 Mar 2003	Drum	53 - 79	02 Nov 2003	92	M
18	Fo	28 Feb – 17 Mar 2003	Drum	53 - 79	02 Nov 2003	96	M
21	Fo	17 Mar 2003	Drum	69 - 74	01 Nov 2003	91	M
33	Fo	17 Mar 2003	Drum	69 - 74	03 Nov 2003	94	M
6	Fo	27 Feb - 03Apr2002	-	58 - 65	20 Oct 2003	172	M
7	Fo	27 Feb – 03 Apr 2002	-	58 - 65	20 Oct 2003	136	F
8	Fo	27 Feb – 03 Apr 2002	-	58 - 65	04 Nov 2003	167	M
10	Fo	27 Feb – 03 Apr 2002	-	58 - 65	-	154	M
12	Fo	27 Feb – 03 Apr 2002	-	58 - 65	-	119	F
5	Fo	16Mar-20 Apr 2002	-	55 - 64	-	139	M
13	Fo	4 Apr 2002	Beach	60	29 Oct 2003	159	M
14	Fo	4 Apr 2002	Beach	60	29 Oct 2003	158	M
16	Fo	16 Mar – 20 Apr 2002	-	55 - 64	02 Nov 2003	125	M
23	Fo	16 Mar – 20 Apr 2002	-	55 - 64	22 Nov 2003	138	F
252	Fr	18 Mar 2004	Drum	75	19 Sep 2003	190	M
253	Fr	28 Feb 2003	Drum	54 - 62	27 Nov 2003	154	F
267	Fr	28 Feb – 17 Mar 2003	Drum	53 - 79	21 July 2004	141	M
266	Fr	22 Apr 2004	Drum	66 - 68	20 June 2005	111	M
167	Fr	28 Feb – 17 Mar 2003	Drum	53 - 79	27 Dec 2004	122	M
265	Fr	22 Apr 2004	Drum	66 - 68	16 June 2005	95	F
264	Fr	22 Apr 2004	Drum	66 -68	16 June 2005	92	F
9	Fo	-	Wild	-	04 Nov 2003	170	F
22	Fo	-	Wild	-	06 Nov 2003	165	M
268	Fr	-	Wild	-	-	360	F

[a] Fo = formaldehyde preserved, Fr = frozen

In addition, the gonads of three wild-caught juvenile *C. chitra* (one frozen, two formaldehyde preserved) that had died during captive rearing were also sexed.

RESULT

The preservation condition of two formaldehyde preserved gonads was not good enough to determine their sex despite histological sectioning. Of the remaining 29 juvenile turtle cadavers, 23 gonads were identified as testes (Fig. 1C, D; Table 1) on the basis of the presence of tubuli seminiferi in the soma and the lack of oogonia, oocytes or follicles. The other six were identified as ovaries because they showed no tubular structures but oogonia, oocytes or follicles (Fig. 1E; Table 1). The overall sex ratio (female: male = 6:23, 79% males) was significantly different from equality (χ^2 = 9.9, $P < 0.01$), demonstrating a heavy male bias in the juvenile cadavers from the captive bred *C. chitra* at Kanchana-

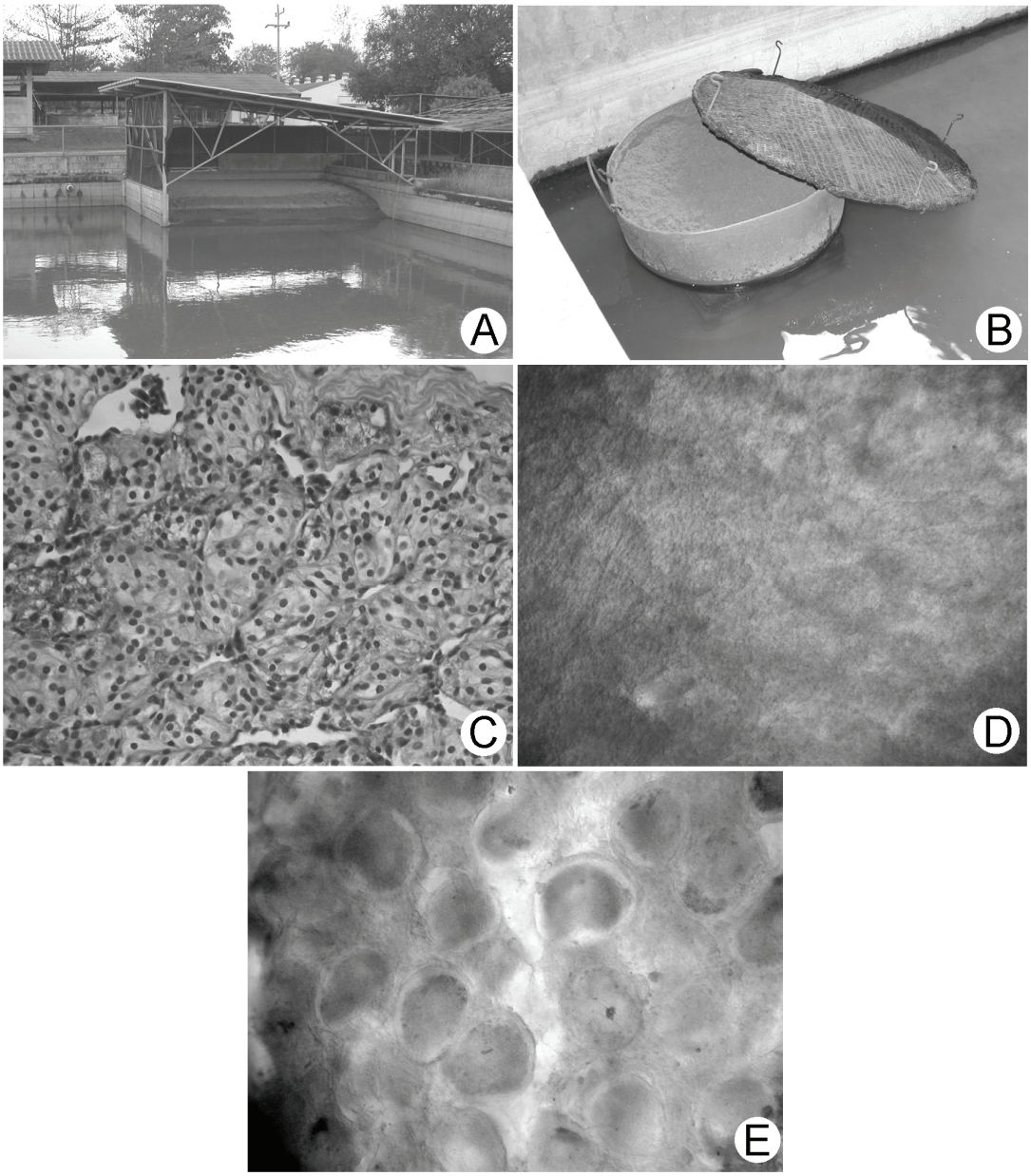

FIGURE 1. A. *C. chitra* breeding pond at KIFDC and artificial nesting beach under the roofed area, "beach" incubation method. **B.** Artificial *C. chitra* egg incubation container at KIFDC in the roofed area, "drum" incubation method. **C.** Juvenile testis from a formalin preserved *C. chitra* specimen (# 259 in table 1), subject to Haematoxylin & Eosin staining after sectioning, (X 600): thin theca testis, soma with tubuli structures containing spermatogonia. **D.** Juvenile testis from formaldehyde preserved *C. chitra* specimen (# 17 in table 1), squash preparation stained with methylene blue (X 600): thin theca testis and soma with tubuli structures recognisable. **E.** Juvenile ovary of formaldehyde preserved *C. chitra* specimen (# 9 in table 1), squash preparation stained with methylene blue (X 600): oocytes and follicles recognisable.

buri. Since GSD should provide an equal sex ratio independently of the incubation environment of the eggs, this result may support an effect of incubation temperature on the sex ratio and possible TSD in *C. chitra*. Although too small a sample size for valid analysis, nevertheless it is interesting that the male biased sex ratio of the turtles that died during development varied with the incubation conditions (Table 1), being highest in the sheltered "beach" (100% male) and decreasing in the "drum" (80% male) and unknown incubation conditions including laboratory (62.5% male).

However, an alternative explanation to TSD for the male biased sex ratio in these captive-bred dead juveniles could be differential developmental mortality of the sexes during incubation, with preferential death of some females, or simply higher mortality of males under captive rearing. For that reason we also sexed the gonads of three wild caught juveniles that had died during captive rearing. Two of those were females and one was a male. Unfortunately this result does not allow statistical evaluation due to the small sample size and sex ratios of wild populations have not been studied.

DISCUSSION

The currently accepted wisdom is that the family Trionychidae has GSD and not TSD, with temperature having no influence on the offspring sex (Ewert and Nelson, 1991; Janzen and Paukstis, 1991; Kuchling, 1999; Seigel and Dodd, 2000; Janzen and Krenz, 2004; Moll and Moll, 2004; Sarre et al., 2004). This assertion is based on the investigation of only three softshell turtle species in the family, that is the Nearctic *Apalone spinifera* and *Apalone mutica* and the Palaearctic *Pelodiscus sinensis*. None of the softshells karyotypically examined show heteromorphic sex chromosomes (Janzen and Paukstis, 1991; Nie et al., 2001), but this may neither be a prerequisite for GSD nor do heteromorphic sex chromosomes necessarily preclude TSD in reptiles (Raynaud and Pieau, 1985). Given the male bias we found in the present study of captive-bred *C. chitra* at KIFDC which died during rearing, it is worth exploring the conclusions in the original studies of those three softshell turtles.

No effect of the egg incubation temperatures on hatchling sex ratios was found in *Apalone spinifera* (Vogt and Bull, 1982). Regarding *A. mutica* the original source states: "data for … *T. muticus* are statistically compatible with 1:1 genetically determined sex ratio … but do not exclude a mild effect of temperature" (Ewert and Nelson, 1991: 54). Although two studies of *Pelodiscus sinensis* consider the species to have GSD (Choo and Chou, 1992; Ji et al., 2003), three other studies concluded that temperature does influence the sex ratio in these species and that the *P. sinensis* has TSD (Zhu and Sun, 2000; Nie et al., 2001; Ran and Yuan, 2004). Although *P. sinensis* does not have heteromorphic sex chromosomes (Nie et al., 2001), a molecular cytogenetic analysis of *P. sinensis* identified ZZ/ZW-type micro-sex chromosomes (Kawai et al., 2007). However, *P. sinensis* has a wide distribution and may represent a cluster of related taxa (Pritchard, 2001, Fritz et al., 2010). Since these four studies of *P. sinensis* were done independently at different locations, it is possible that either different taxa (currently not considered valid or unrecognised) or cryptic variants within a taxon were studied that differ in their mode of sex determination. Alternatively, the balance between TSD and GSD may vary within a species and be

differentially susceptible to environmental (experimental) conditions.

Thus, apart from the fact that only three out of 26 trionychid species (Engstrom et al. 2004) from temperate to subtropical zones and none from the tropics have been studied, the results and conclusions for at least the only Old World species (*P. sinensis*) are ambiguous rather than clear cut regarding the influence of incubation temperature on hatchling sex ratios. Phylogenetically TSD appears to be the ancestral mechanism of sex determination in turtles and has been lost at least six times in various groups, including in the family Trionychidae, but not in their sister group, the family Carettochelyidae (Janzen and Krenz, 2004). Of the two subfamilies, Cyclanorbinae and Trionychinae, in the family Trionychidae, so far sex determination has been studied in only three species belonging to the Trionychinae. According to Engstrom et al. (2004), the genus *Chitra* belongs to a basal sister group (Gigantaestuarochelys) to the groups which include the two species with proven GSD (Apalonina) and the one species with questionable GSD or TSD results (Amydona) in the subfamily Trionychinae. It has not yet been investigated at which node in the phylogeny of the Trionychidae that TSD has been lost and replaced with GSD. The present results regarding *C. chitra* suggest that the replacement of TSD with GSD could have taken place after the phylogenetic split of the more advanced Trionychynae with GSD from the basal group containing *Chitra*. Apart from this possibility, GSD and TSD in reptiles should not be seen as a dichotomy, but as the ends of a continuum of states represented by (i) species whose sex is determined primarily by genotype, (ii) species where genetic and environmental mechanisms coexist and interact in lesser or greater measure to bring about sex phenotypes, and (iii) species where sex is determined primarily by the temperature of incubation (Raynaud and Pieau, 1985; Pieau, 1996; Sarre et al., 2004).

Unfortunately, this complexity in sex determination mechanisms of reptiles generally, and of softshell turtles specifically, does not seem to be widely recognised in the turtle conservation community. For example, in the "Turtle Conservation" book chapter "Manipulation of turtle populations for conservation" Seigel and Dodd (2000: 225) simply state: "… Trionychidae … have true genotypic sex determination". Wildlife managers should never rely on such academic wisdom for the purpose of planning breeding protocols and recovery strategies for critically endangered species without considering the possible alternative; namely that temperature may influence sex ratios through either sex determination or differential survival. In particular, since it is known that different species in other turtle families can exhibit either TSD or GSD (Ewert and Nelson, 1991; Ewert et al., 2004; Janzen and Krenz, 2004), the absence of published evidence for TSD in a particular species (in our case *C. chitra*) should never be taken as evidence that incubation temperature does not influence sex ratios in this species, even if it belongs to a family (Trionychidae) generally believed to have GSD.

This message is potentially reinforced by our finding of a male biased sex ratio in the captive bred *C. chitra* that died during captive rearing at Kanchanaburi. It would not be ideal for the recovery of *C. chitra* if this sub-sample was representative of the 710 captive bred turtles produced in the six years prior to this study and of the 79 of them which were already released into the

wild (Jon River) on 22 June 2005 to reinforce the dwindling wild population. Release stock with a balanced or even a female biased sex ratio would have been preferable in such a recovery program for a critically endangered species (Seigel and Dodd, 2000).

Heavy male biased sex ratios in turtle conservation programs are not without precedence. For example, prior to the 1980s and the understanding of TSD in sea turtles, incubation of sea turtle eggs in Styrofoam boxes in sheds was popular in field conservation projects because it was a simple way of protecting the eggs and gave high hatch rates. Because such boxes tend to be cooler than the natural nests on the sand beaches, this leads to extreme male-biased sex ratios being introduced into many sea turtle conservation programs around the world (Seigel and Dodd, 2000; Wibbels, 2003). Unfortunately, *C. chitra* is much rarer and more threatened than most of the other still widespread sea turtles, meaning that there is much less room for conservation mistakes without reducing the survival prospects of the species. This also imposes upon the captive breeding stock, in that it is traditionally founded by a low number of captives and so prone to severe genetic bottlenecks. In addition, if sex determination is a balance between TSD and GSD, the founder stock may not represent the ideal, for that locality or the one to be released into, or complete range of the wild population. No matter if or how much genetics plays a role in sex determination of *C. chitra* (GSD and TSD can be viewed as the ends of a continuum: Sarre et al., 2004), the results presented in this study indicate the possibility that the incubation methods under roofed areas used in the *C. chitra* breeding program at Kanchanaburi had a masculinising effect on captive bred offspring, similar to the Styrofoam box incubation of sea turtle eggs in sheds. However, differential mortality could not be discriminated from TSD as a cause for the male biased juvenile cadavers in this study. Unfortunately a number of additional juvenile *C. chitra* that had died during captive rearing at KIFDC could not be sexed due to their poor preservation status and macerated gonads. Further experiments are necessary and currently under way to investigate the possibility that incubation environment affects sex ratios in *C. chitra*.

Like numerous other large bodied river turtles in a variety of taxonomic groups, *C. chitra* nests during the low water season on sand banks with no or sparse vegetation cover, with nest sites often exposed to the full sun. Hatchling sex ratios in TSD species are determined by a complicated mixture of factors which affect the nest temperatures, such as the female size and the resulting nest depth, degree of shading and nest orientation (i.e., north-facing slope versus south-facing, etc.). As a result of these variables natural nests of species with TSD may produce either all female, or all male, or a mixed gender sex brood depending upon the temperature gradient from the top to the bottom of the nest chamber (Moll and Moll, 2004). The constant temperature producing a 1:1 sex ratio in TSD species is called the threshold or pivotal temperature, with female turtles being predominantly produced at temperatures higher than the pivotal temperature, and males at lower temperatures (but see above for females in TSD II). The large sand bank nesting river turtles with TSD seem to have the highest pivotal temperatures of all chelonians investigated, such as 32.5 °C for the giant South American river turtle *Podocnemis expansa* (Valenzuela, 2001), which has a body size comparable to that of *C. chitra*,

and between 31 – 32 °C for the slightly smaller *Podocnemis unifilis* (Souza and Vogt, 1994). At Kanchanaburi, *C. chitra* females had only shaded nesting beaches available and artificial incubation also only took place indoors or under roofs providing constant shade (Fig. 1A, B), as detailed before (Kitimasak et al., 2003). Even though the resulting mean incubation temperatures of approximately 29 - 31 °C (Kitimasak et al., 2003) would be in the female-producing range of many turtle species, they were cooler than those in natural nests on sun exposed sandbanks that typically produce females in large South American sandbank nesting river turtles (< 31.3°C: Souza and Vogt, 1994; < 32.5°C : Valenzuela, 2001). Interestingly, one study found that for the softshell turtle *P. sinensis*, the predominantly female producing temperatures were also high at 33 and 35 °C (Nie et al., 2001). If the incubation temperature influences the sex ratio in *C. chitra*, a possibility indicated by our data, the male bias in the dead captive bred *C. chitra* at Kanchanaburi would not be surprising as it would reflect the relatively low incubation temperatures.

To further evaluate the possibility of TSD in *C. chitra* and to allow future adjustment of sex ratios in the captive breeding program at Kanchanaburi, from 2007 onwards part of each egg clutch have been incubated under controlled, constant temperatures. Because of the critically endangered status of *C. chitra* the standard method of sacrificing hatchlings for sexing in such experiments will not be used. Rather, non-lethal sexing will be done later in head started live juveniles by endoscopy instead (Kuchling and Kitimasak, 2009).

ACKNOWLEDGEMENTS

This study was funded by the Turtle Survival Alliance (TSA) through a grant from The Batchelor Foundation. We thank R. Hudson and J. Murray for organisational help; S. Ferrell for help with some post mortem dissections; and Prof. N. Chansue and Prof. A. Sailasuta, Chulalongkorn University, for help with histology.

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