RELOCATING GREEN TURTLE (*CHELONIA MYDAS*) EGGS TO OPEN BEACH AREAS PRODUCES HIGHLY FEMALE-BIASED HATCHLINGS

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Abstract.—Due to the effect environmental temperature has on the development of sea turtle embryos, relocating egg clutches can threaten incubation success and skew natural sex ratios. Even small rises in nest temperature due to mismanaged hatchery efforts and global climate change may lead to the progressive feminization of already endangered sea turtle populations. Our study measured and compared the incubation temperature and estimated hatchling sex ratios of natural and relocated Green Turtle (*Chelonia mydas*) clutches at Chagar Hutang beach, Redang Island, and clutches relocated into a protective hatchery site at Tiga Ruang beach, Perhentian Islands, in Terengganu, Malaysia. We found that relocating clutches raised incubation temperature by 1.8° C and were estimated to have increased the ratio of female hatchlings produced by 45% compared to clutches that were left to incubate under natural conditions. Sex ratio estimates were verified by the histological examination of gonad tissue from deceased hatchlings and confirmed our prediction of highly female-biased hatchling sex ratios. If such practice continues, this lack of male hatchling production will cause the feminization of the population and thus the future extinction of the already threatened populations of nesting sea turtles in the region. We recommend the use of natural palm frond materials to artificially cool relocated clutches at the remote open beach hatchery site and the continued monitoring of natural and relocated nest temperatures to estimate hatchling sex ratios predicted from this region.

Key Words.—artificial cooling; conservation; endangered species; gonad histology; sex ratio; South China Sea

INTRODUCTION

Sea turtle populations around the world have experienced dramatic declines over the past several decades due to mounting anthropogenic threats including; the loss of nesting and foraging habitat due to coastal development and environmental degradation, being caught in nets or hooked as by-catch in the fishing industry, ingestion or entanglement in floating plastic debris, being hunted for their meat and ornamental shells, and the harvesting of turtle eggs from nesting beaches for human consumption (Chan 2006; Hamann et al. 2006; Jensen et al. 2006; Pilcher et al. 2010; Pfaller et al. 2020). Due to these pressures, the protection and management of sea turtle rookeries and the production of as many high-quality hatchlings as possible have become the primary focus of conservation efforts worldwide (García et al. 2003). One strategy used to protect sea turtle eggs in areas where natural nests are vulnerable to poaching, high rates of natural predation, tidal inundation, or coastal erosion, is the relocation of egg clutches from their natural nesting site into a protective hatchery (García et al. 2003; van de Merwe et al. 2005). There are conflicting opinions amongst conservationists, however, regarding the benefits of relocating eggs versus leaving clutches to incubate *in situ* at their natural nest site. Several studies have indicated that relocating turtle eggs, with proper hatchery management, can increase hatching success and in turn increase the potential number of offspring recruited into threatened or endangered populations (García et al. 2003; Dutton et al. 2005). Nevertheless, there is still concern that changes in the nest environment due to clutch relocation, especially when relocated into an unshaded, open-beach hatchery, may increase incubation temperature and skew natural hatchling sex ratios (Chan and Liew 1995; Pintus et al. 2009; Jensen et al. 2016).

Hatcheries were first established within Malaysia following alarming declines in sea turtle nesting trends across the region (Chan 2006). The practice of relocating egg clutches originally laid under the shade of natural vegetation into unshaded open-beach hatcheries inevitably raised incubation temperatures and resulted in lower hatching success and highly female-biased hatchling sex ratios (Chan and Liew 1995). An assessment by Colin J. Limpus (unpubl. report) concluded that rookeries in Malaysia had produced predominantly female hatchlings between 1961 and 1993, and the genetic analysis of juvenile Green Turtles

(*Chelonia mydas*) found at two major Malaysian foraging grounds indicates a current 4:1 female-biased population, which Jensen et al. (2016) believe largely reflects the decades of use of unshaded beach hatcheries at Malaysian rookeries. Although the methodologies and timeframe of different studies carried out at nesting sites around the world vary, the tendency of female-biased hatchling production is accepted to be globally predominant (Wibbels 2003; Jribi et al. 2013; Hays et al. 2014; Booth et al. 2020).

The sex of sea turtle hatchlings is determined during incubation through a process termed temperature-dependent sex determination, in which the temperature during the middle third of embryonic development, known as the thermosensitive period (TSP), directs gonadal differentiation in the developing embryos (Georges et al. 1994; Broderick et al. 2000; Godfrey and Mrosovsky 2006; Kobayashi et al. 2017). During the TSP both male and female sexes are produced within a narrow range of incubation temperature, between 28°–31° C, as incubation temperature increases within this zone, the resulting sex ratio of hatchlings shifts from all-male to all-female (Yntema and Morsovsky 1980; Jourdan and Fuentes 2015; Gammon et al. 2020), and outside of this range, only single sexes are produced (Ackerman 1997). Many factors can influence the incubation temperature of sea turtle nests, including seasonal changes in air temperature and rainfall to nest site-specific factors such as the geographic orientation of a beach, variation in sand texture and color, and whether the nest site is under direct sunlight or shaded by vegetation (Bustard and Greenham 1968; Mrosovsky 1994). Additionally, the depth of a nest, along with microenvironmental factors such as moisture and the metabolic heat generated by developing embryos can affect the nest temperature (Bustard and Greenham 1968; Broderick et al. 2000; Sifuentes-Romero et al. 2018). Extended periods of high nest temperatures can result in higher rates of embryonic mortality, as well as an increase in the frequency of morphological abnormalities like carapace scute mutations in the resulting hatchlings (Jourdan and Fuentes 2015; Tanabe et al. 2021). Incubation temperature also influences sea turtle hatchling size, their energy reserves, self-righting ability, and their crawling and swimming speed during the early dispersal period (Burgess et al. 2006; Maulany et al. 2012; Rusli et al. 2015; Booth 2017; Suhaimi et al. 2020). The ability for hatchlings to avoid predation directly depends upon their locomotor performances as they emerge from their nest to crawl and swim past a gauntlet of both terrestrial and marine predators before reaching the relative safety of the open ocean (Ischer et al. 2009; Rusli et al. 2015; Booth 2017).

Concerns over the impact of rising global temperatures on the reproductive success of sea turtle species have heightened the need for conservation management strategies, like artificial nest cooling, to mitigate the potential threats associated with high incubation temperatures (Hays et al. 2003; Hawkes et al. 2007; Fuentes et al. 2009; Hamann et al. 2010; Wood et al. 2014). Projected increases in global temperature could result in the production of highly female-biased hatchlings (Fuentes et al. 2009), which in turn could lead to decreased rates of fertilization and loss of genetic variation, further jeopardizing the ability of endangered sea turtle populations around the world to adapt to climate change (Hamann et al. 2010). Although the breeding frequency and polygamous mating behavior of male turtles may help mitigate the effects of female-biased hatchling production at a specific rookery, it is still unknown how many or what proportion of males in a population are sufficient to sustain, let alone recover, threatened sea turtle populations (Hays et al. 2017; Jensen et al. 2018).

Historically, researchers investigated sex ratios of sea turtle hatchlings by incubating egg clutches in laboratories at constant temperatures, then using gonad histology to determine hatchling sex, which required the killing of large groups of hatchlings to determine how incubation temperature influenced the resulting sex (Yntema and Mrosovsky 1980; Palaniappani et al. 2000; Godfrey and Mrosovsky 2006). Although gonad histology is the most accurate method for determining the sex of sea turtle hatchlings (King et al. 2013; Staines and Booth 2019), due to the endangered status of sea turtle populations, researchers today estimate hatchling sex-ratios through non-invasive proxies (Girondot et al. 2010; Wyneken and Lolavar 2015; Fuentes et al. 2017). Primarily, researchers deploy temperature data loggers within the sand of a nesting beach or inside of an incubating clutch of eggs to calculate nest temperature and derive sex ratio predictions (Broderick et al. 2000; King et al., 2013; Tapilatu and Ballamu 2015; Staines and Booth 2019). Additionally, determining the incubation duration of a nest can be an effective method for estimating the hatchling sex ratio (Godley et al. 2001), as sea turtle nests that experience higher incubation temperatures have shorter incubation periods and thus, are indicative of female-biased hatchling sex ratios (Broderick et al. 2000; Godley et al. 2001). It is recommended that these proxies should be validated with actual sex ratios expressed in nests and that caution is necessary when interpreting sex ratio estimates from proxies, particularly as using incubation temperature alone can underestimate male hatchling production in these circumstances (Girondot et al. 2010; Wyneken and Lolavar 2015; Fuentes et al. 2017).

Our study investigated the influence that relocating Green Turtle clutches has on incubation temperature and resultant hatchling sex ratios at two important nesting

sites in Peninsular Malaysia: Chagar Hutang and Tiga Ruang turtle sanctuaries, located on islands off the coast of Terengganu State. No studies to date have investigated the incubation temperatures of clutches relocated inside the hatchery at Tiga Ruang or the resulting sex ratios of hatchlings produced there. Artificially constructed nests may have different microenvironments compared to the original natural nest, including temperature, which regulates and influences embryonic development. Therefore, it is crucial to compare the nest temperatures and the resulting sex ratios of hatchlings produced between relocated clutches and those incubated at their natural site to better replicate natural incubation temperatures that produce high-quality hatchlings at optimal sex ratios.

MATERIALS AND METHODS

Study sites.—The two study sites, Chagar Hutang Turtle Sanctuary on Redang Island (5º48.778'N, 103º0.502'E) and Tiga Ruang Turtle Sanctuary in the Perhentian Islands (5º54.395'N, 102º45.945'E), lie 32 km apart on opposite ends of a small tropical archipelago 20–30 km off the northeast coast of Terengganu, Malaysia (Fig. 1). Chagar Hutang is one of the most important nesting beaches for Green Turtles in the region and is managed by Sea Turtle Research Unit (SEATRU) staff and rangers. Between 700 to 1,500 clutches are laid there annually (Chan 2013), with the majority of nests left to incubate naturally (Fig. 2). Egg clutches are only relocated at this site if the original nest location is deemed unsuitable for incubation; for example, clutches laid along sections of the beach at risk of erosion or inundation by freshwater streams and ocean high tides. In the Perhentian Islands, illegal poaching threatens

sea turtle nests left unguarded. Therefore, the local Department of Fisheries (DoF) staff relocates any nests encountered into a protective hatchery at Tiga Ruang (Fig. 2). The 8 × 4 m fenced hatchery is constructed in the same location each year and positioned 2 m from the high tide line, 1 m from the vegetation line on the inland side, and is partially shaded by natural tree canopy in the late afternoon. Between 100 to 300 nests are relocated into the Tiga Ruang hatchery each year.

Sand and nest temperatures.—We measured sand and nest temperatures with Thermochron iButton data loggers (model DS1922L; Maxim Integrated, San Jose, California, USA). These data loggers have a memory size of 2KB and can record temperature with an accuracy of ± 0.5° C over a range of -10° to 65° C. We programmed data loggers to record temperature every hour and buried them in the center of a nest during natural oviposition or during the relocation of egg clutches into artificially constructed nests at both sites between May and September 2019. We randomly selected experimental nests throughout this study to coincide with the peak of the nesting season, which occurs from June to August in this region. We placed data loggers into six natural nests and six relocated nests along the length of Chagar Hutang beach, and into 10 nests relocated into the Tiga Ruang hatchery site. We were unable to measure natural nests at Tiga Ruang because all nests discovered were relocated into the hatchery due to the threat of poaching in the islands. We left data loggers in place within the nests to record temperature over the entire course of incubation. We calculated incubation duration as the number of days from when eggs were first laid until the first emergence of hatchlings from a nest. Two to three days after the emergence of hatchlings, we excavated
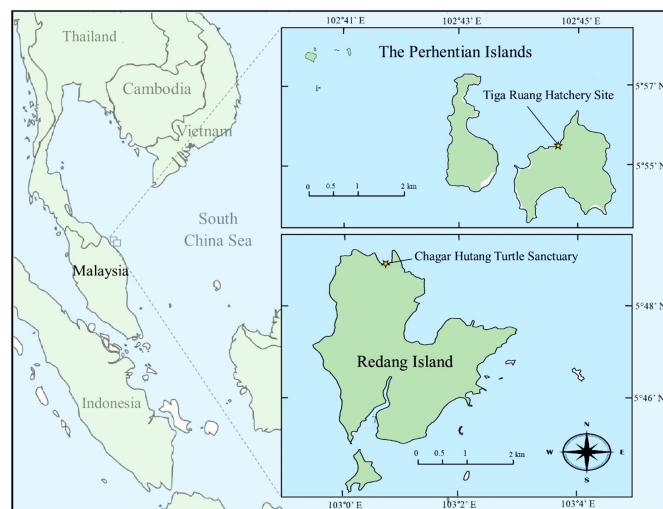

FIGURE 1. Map of Southeast Asia indicating the locations of Chagar Hutang and Tiga Ruang Turtle Sanctuaries on Redang and the Perhentian Islands off the East coast of Peninsular Malaysia. (Adapted from Google Maps).
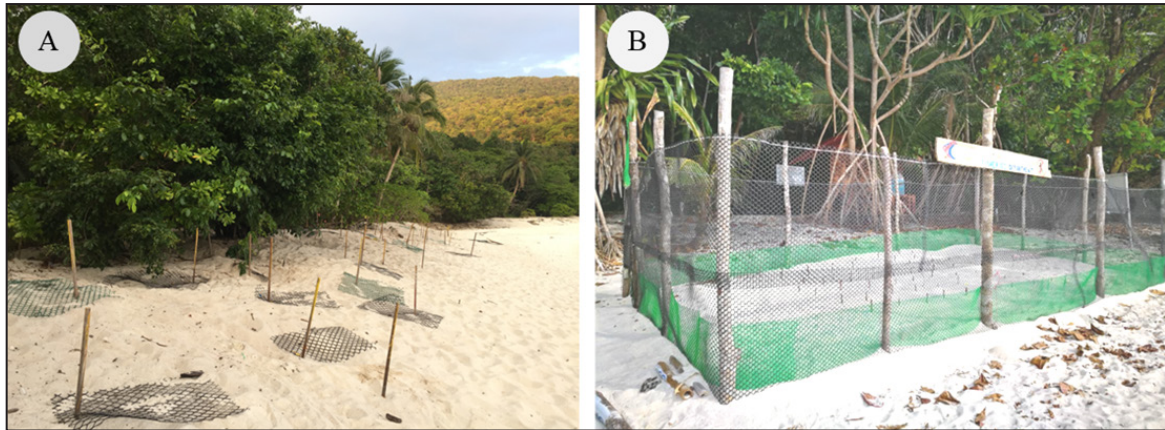
Figure 2. (A) Natural and relocated Green Turtle (*Chelonia mydas*) clutches incubating at Chagar Hutang Turtle Sanctuary, marked with a stick and covered with plastic mesh to prevent predation by monitor lizards. (B) Relocated clutches inside the hatchery site at Tiga Ruang Turtle Sanctuary, fenced off to prevent egg poaching. (Photographed by Nicholas Tolen).

the nests to retrieve the data loggers and download their data. We measured nest depth and recorded the number of unhatched eggs, dead hatchlings, and live hatchlings in each nest to calculate hatching success: ([clutch size - unhatched eggs] / clutch size) × 100; and emergence success ([clutch size - unhatched eggs - live-in-nest hatchlings] / clutch size) × 100 for each clutch (Kobayashi et al. 2017). Additionally, we buried three data loggers at a depth of 40 cm along the length of Chagar Hutang beach in the middle of the region where natural nests were constructed, and two data loggers along the centerline of the Tiga Ruang hatchery. We replaced sand temperature loggers every 2 mo to ensure their memory was not filled. We used hourly nest temperature measurements to calculate the average nest temperature and the average temperature during the TSP.

Estimating hatchling sex-ratios.—We calculated the percentage of female hatchlings for each experimental nest by averaging the hourly nest temperature measurements during the TSP following the methods of Reboul et al. (2021). We used a constant temperature degree algorithm developed by Booth and Freeman (2006) for the southern Great Barrier Reef (GBR) Green Turtle population to calculate the hypothetical hourly increments of embryonic growth from nest temperature measurements. This algorithm was used to determine the average temperature of an experimental nest during its TSP, calculated as the mean nest temperature during the middle third of incubation. We then used a logistical equation that describes the relationship between sex ratio and mean TSP temperature to estimate the percentage of female hatchlings for each nest (Booth and Freeman 2006), but the curve was shifted by 1.0° C to account for a one-degree difference between the pivotal temperature of the southern GBR population (28.1° C; Miller and

Limpus 1981) and the proposed pivotal temperature (29.1° C) for the Malaysia Green Turtle population (Chan and Liew 1995; van de Merwe et al. 2006; Reboul et al. 2021).

Hatchling sex ratio using gonad histology.—We collected 77 dead hatchlings from nine nests without data loggers and one nest with a data logger placed inside of it at the Tiga Ruang hatchery. We immediately dissected the left and right kidney-gonad tissue complexes free from the dead hatchlings and placed them into specimen jars containing 70% ethanol for transportation back to the laboratory where they were then transferred into 10% neutral buffered formalin. We processed the tissue samples following the standard histological procedures for reptilian gonad tissue (Whiteley et al. 2018) and determined the sex of each hatchling based on ovary and testes cellular characteristics previously described for Green Turtles (Yntema and Mrosovsky 1980; King et al. 2013; Sari and Kaska 2016). Those authors found the cortices of ovary tissue (Fig. 3) were noticeably thicker, consisting of multiple layers of columnar cells, in contrast to the thinner single layer of flattened squamous cells identified as testes tissue (Fig. 3), and that a dense and disorganized medulla characterized ovaries while the presence of seminiferous tubules in the medulla characterized testes (Yntema and Mrosovsky 1980). The paramesonephric ducts of female hatchlings were characterized by organized lumen tissue lined by a ring of epithelial columnar cells and attached to kidney tissue by a long and thin stalk, whereas the paramesonephric ducts of male hatchlings, were attached by a short and thick stalk and characterized by disorganized and disintegrated lumen tissue (Fig. 3), as described by Sari and Kaska (2016). We calculated the percentage of female hatchlings as the number of female hatchlings identified out of the total number of dead hatchlings examined multiplied by 100.
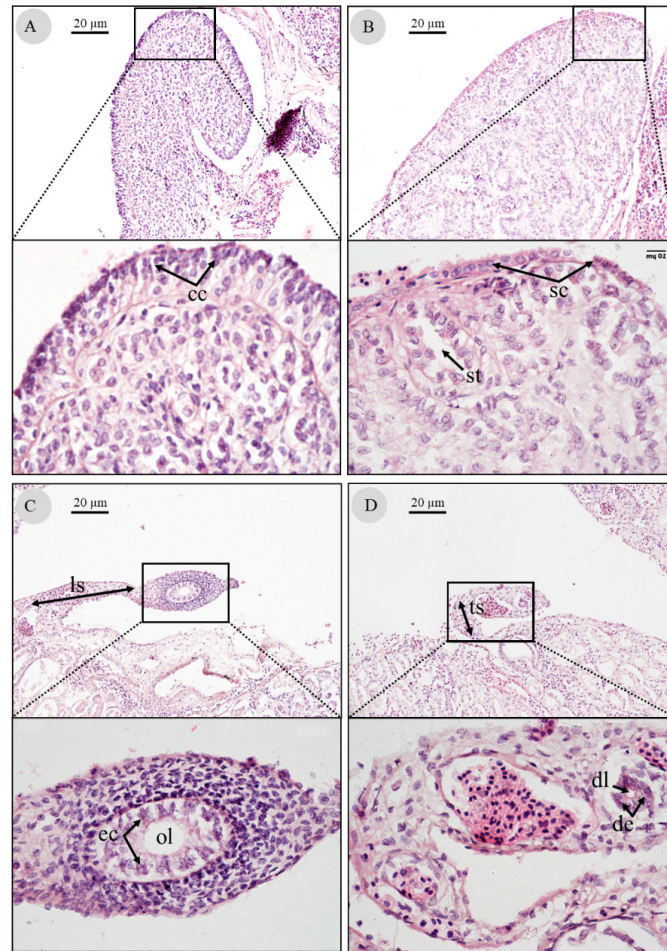
FIGURE 3. Gonad histology of deceased neonate Green Turtle (*Chelonia mydas*) hatchlings shows that (A) Ovary tissue is characterized by a thick cortex of columnar cells (cc) and a dense disorganized medulla lacking seminiferous tubules. (B) Testis tissue is characterized by a thin cortex of flattened squamous cells (sc) and a medulla populated by seminiferous tubules (st). (C) The paramesonephric duct of female hatchlings is characterized by organized lumen tissue (ol) lined with epithelial columnar cells (ec) attached to the kidney by a long thin stalk (ls). (D) In male hatchlings it is characterized by a short and thick stalk (ss) with disintegrated epithelial (de) and disorganized lumen tissue (dl). Slides were stained using H & E procedures and photographed at 200× magnification, insets 400× magnification. (Photographed by Nicholas Tolen).

Statistical analysis.—We performed all statistical analyses using IBM SPSS software (IBM Corp, Armonk, New York, USA). We analyzed nest depth, incubation duration, mean incubation temperature, mean TSP temperature, hatching success, nest emergence success, and the estimated percentage of female hatchlings using One-way Analyses of Variance (ANOVA) with incubation method (natural at Chagar Hutang, relocated at Chagar Hutang, and relocated at Tiga Ruang) as fixed factors. We conducted a Shapiro-Wilk Test to ensure data was normally distributed, while Levine's Test was used to test for homogeneity of variance, for which our data met this assumption. We used Arc Sine transformed hatching success, nest emergence success, and estimated percentage of female hatchling data to conform data sets to assumptions of normality. We used Pearson's correlation to determine and significant correlation between variable data sets. We conducted a Mann-Whitney U test to determine whether there was a significant difference between sex ratio estimates and hatchling gonad histology. We assumed the significance for all tests at $P < 0.05$. Mean values are shown with ± 1 standard deviation (SD).

RESULTS

Sand and nest variables.—We observed < 0.5° C difference in daily ambient sand temperatures between Chagar Hutang beach and the Tiga Ruang hatchery, allowing us to compare incubation methods between the two study sites. We found that natural nests had, on average, lower mean incubation temperatures and mean
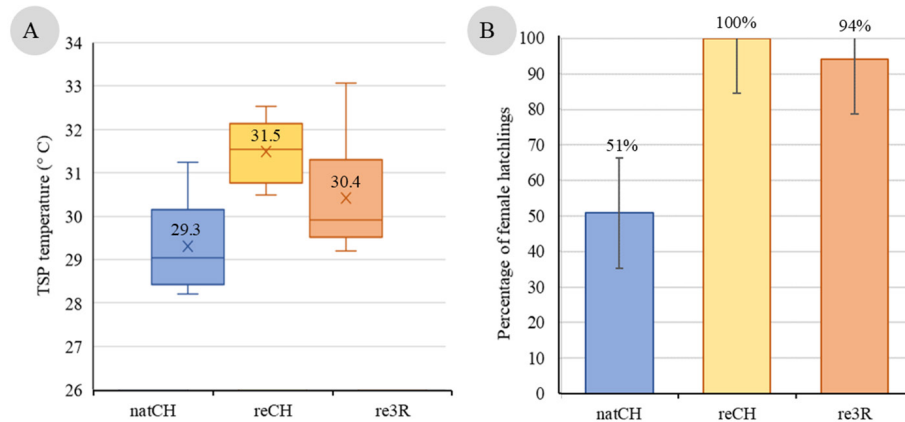
FIGURE 4. (A) Box and Whisker plot of mean temperature during the temperature sensitive period (TSP) and (B) bar graph of the predicted percentage of female Green Turtle (*Chelonia mydas*) hatchlings produced from natural nests at Chagar Hutang (natCH), relocated nests at Chagar Hutang (reCH), and relocated nests at the Tiga Ruang hatchery (re3R) in Malaysia.

TSP temperatures (Fig. 4) than relocated nests at both Chagar Hutang beach and the Tiga Ruang hatchery site (Table 1). We found that mean incubation temperatures were 2.2° C warmer in relocated nests at Chagar Hutang beach (31.4° ± 0.8° C) and 1.2° C warmer in relocated nests at Tiga Ruang hatchery (30.4° ± 1.1° C) compared to natural nests recorded at Chagar Hutang beach (29.2° ± 1.1° C). Overall relocated nest temperatures measured at both Chagar Hutang and Tiga Ruang averaged 31.0° ± 1.1° C, 1.8° C higher than the mean temperature of nests left to incubate under natural conditions. In addition, we noted that relocated nests at the Tiga Ruang hatchery had significantly shallower depths than both natural and relocated nests at Chagar Hutang beach, which had relatively similar depths (Table 1). We observed a mean increase in incubation temperature of 1.4° ± 0.8° C during the final third of development, and this rise was similar in relocated and naturally incubated clutches.

Incubation duration, hatching, and emergence success.—We found that the mean incubation period (51 ± 2.79 d) of 22 nests was strongly negatively correlated $(r = -0.79, t = 3.85, df = 20, P = 0.001)$ to mean incubation temperature (30.5° ± 1.31° C). Therefore, we included mean incubation temperature as a covariate for incubation period, in which incubation method had a significant effect (Table 1). We found that natural nests had the longest incubation periods lasting up to 58 d. We determined that mean incubation temperature was not significantly correlated $(r = 0.05, t = 0.23, df = 20, P = 0.815)$ to mean nest depth (69.41 ± 16.56 cm) for 22 nests, and the mean increases in nest temperature associated with metabolic heat generated during incubation (1.42° ± 0.81° C) was not correlated $(r = 0.37, t = 1.86, df = 20, P = 0.07)$ to clutch size (95.36 ± 20.89 egg). We determined the incubation method did not affect hatching success or emergence success (Table 1).

Estimated hatchling sex ratios.—Our sex ratio estimates indicate that incubation method affected the percentage of female hatchlings emerging from nests (Fig. 4). We predicted that all the relocated nests monitored at Chagar Hutang beach produced 100 ± 0% female hatchlings and that relocated nests monitored at Tiga Ruang hatchery produced 94.3 ± 10.2% female hatchlings (Table 1), with eight of 10 nests producing highly female-biased hatchlings (> 90% female). In contrast, only two of the six natural nests at Chagar Hutang beach were predicted to have highly female-biased hatchlings, with the average of all monitored nests producing 52 ± 43.0% female hatchlings over a range of 1–100% (Fig. 5).
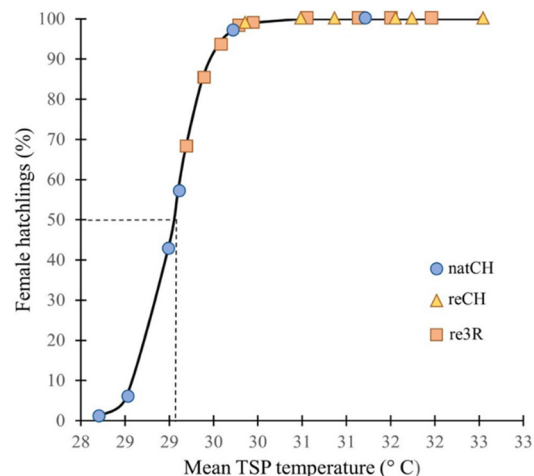

FIGURE 5. Fitted sex ratio curve depicting the estimated percentage of female hatchlings calculated from incubation temperatures of both natural (nat) and relocated (re) nests at Chagar Hutang (CH) beach and the Tiga Ruang (3R) hatchery, Malaysia. The dotted line represents the pivotal temperature (29.1° C) proposed for the Malaysian Green Turtle (*Chelonia mydas*) population, the incubation temperature that would hypothetically produce equal ratios of male and female hatchlings.

TABLE 1. Descriptive statistics for Green Turtle (*Chelonia mydas*) clutches incubated under three incubation conditions: natural sites at Chagar Hutang (natCH), relocated sites at Chagar Hutang (reCH), or relocated sites at Tiga Ruang (re3R). Comparison between conditions were tested using Analysis of Variance (ANOVA) or Analysis of Covariance (ANCOVA), incubation period with incubation temperature as a covariate. Significant results ($P < 0.05$) are marked with an asterisk (*) and the results of an unequal n HSD post-hoc test are described. Sample size is n and standard deviation is SD.

	Incubation condition	n	Mean ± SD	Range	Comparison between conditions (test statistic, *P*-value, test results)
Nest depth (cm)	natCH	6	80.7 ± 9.8	70–94	$F_{2,19} = 38.781$ $P = 0.001*$ natCH ≈ reCH > re3R
	reCH	6	84.5 ± 6.6	75–93	
	re3R	10	53.6 ± 7.0	41–64	
Incubation duration (days)	natCH	6	53.5 ± 3.2	49–58	$F_{2,19} = 8.160$ $P = 0.003*$ reCH > re3R > natCH
	reCH	6	48.7 ± 1.2	47–49	
	re 3R	10	50 ± 1.8	47–50	
Incubation temperature (° C)	natCH	6	29.2 ± 1.1	28.25–31.14	$F_{2,19} = 6.948$ $P = 0.005*$ reCH > re3R > natCH
	reCH	6	31.4 ± 0.8	30.36–32.41	
	re3R	10	30.7 ± 1.1	29.38–32.97	
TSP (° C)	natCH	6	29.3 ± 1.1	28.20–31.21	$F_{2,19} = 6.119$ $P = 0.009*$ reCH > re3R > natCH
	reCH	6	31.5 ± 0.7	30.49–32.53	
	re3R	10	30.4 ± 1.2	29.29–33.06	
Metabolic heat generated (° C)	natCH	6	1.3 ± 1.0	0–2.50	$F_{2,19} = 0.171$ $P = 0.844$ natCH ≈ reCH ≈ re3R
	reCH	6	1.5 ± 0.8	0–2.25	
	re3R	10	1.5 ± 0.8	0–2.25	
Hatching success (%) (*ArcSin transformed*)	natCH	6	83 ± 0.03	79.4–86.7	$F_{2,24} = 0.489$ $P = 0.619$ natCH ≈ reCH ≈ re3R
	reCH	6	82 ± 0.05	73.6–86.1	
	re3R	15	77 ± 0.1	53.9–96.2	
Emergence success (%) (*ArcSin transformed*)	natCH	6	81 ± 3.7	76.5–86.7	$F_{2,24} = 0.961$ $P = 0.397$ natCH ≈ reCH ≈ re3R
	reCH	6	80 ± 4.3	72.1–84.3	
	re3R	15	72 ± 17.1	30.2–95.2	
Female hatchlings (%) (*ArcSin transformed*)	natCH	6	52 ± 43.0	1.2–100	$F_{2,19} = 8.515$ $P = 0.002*$ natCH > re3R > reCH
	reCH	6	100	100	
	re3R	10	94 ± 10.2	68.4–100	

Hatchling gonad histology.—We found that 97% (74 of 77) of the dead hatchlings collected from the Tiga Ruang hatchery site were female (Table 2). Only one of the 10 nests we sampled produced male hatchlings, although the sample size was < 10 for most nests. Even in the one nest that produced two males, the percentage of males (29%) was low. The percentage of female hatchlings predicted using sex ratio estimates (94.3 ± 10.2%) and gonad histology (97.1 ± 9.1%) were not significantly different (U = 33.0, *P* = 0.218). In the nest that had a temperature trace record, all six dead hatchlings were female, with the nest temperature trace predicting 97% female hatchling production.

DISCUSSION

Nest temperature and estimated sex ratio.—In this study, we determined that relocating clutches of Green Turtle eggs at both the Chagar Hutang and Tiga Ruang Turtle Sanctuaries raised incubation temperature on average by 1.8° C and were estimated to have increased the percentage of female hatchlings produced by 45% compared to nests that were left to incubate under natural conditions. Additionally, we predicted the majority of relocated nests in our study would produce only female hatchlings. Mean TSP temperatures for relocated nests at Chagar Hutang beach averaged 31.5° C, with each recorded nest predicted to produce 100% female hatchlings, and even in those relocated nests that we predicted to produce some male hatchlings, the percentage of males was relatively low. We suggest future management practices at these hatcheries are changed to ensure an adequate proportion of male hatchlings (> 25%; unpubl. data) are produced.

The results from our study suggest that when the staff at Chagar Hutang beach relocated at-risk clutches, they

Table 2. The sex identification of gonad tissues of dead Green Turtle (*Chelonia mydas*) hatchlings collected (n) from relocated nests at the Tiga Ruang hatchery site, Malaysia. The sex of one hatchling from nest number 76 was unable to be determined due to the decomposition of the specimen. The symbol % = percentage and the abbreviation NI = number of identified.

Nest number	n	% clutch examined	NI females	NI males	% identified females
76	14	13.2	13	—	100
84	10	15.6	10	—	100
101	4	5.1	4	—	100
104	4	4.5	4	—	100
124	7	6.9	5	2	71.4
138	14	15.1	14	—	100
282	4	10.3	4	—	100
283	6	13.0	6	—	100
292	4	4.5	4	—	100
296	10	15.9	10	—	100
Total:	77	10.9	74	2	97.1

chose open and unshaded sections of the beach along the perimeter of naturally deposited nests, away from the partial to full shade provided by overreaching natural vegetation, resulting in high incubation temperatures. The bias in artificial nest placement may be due to the experience of rangers with relocating nests along the open beach, which results in shorter incubation periods and a lower risk of nest predation (Najwa-Sawawi et al. 2021). Turtle nests located in the shade along the edge of forests are thought to be more susceptible to predation by Asian Water Monitors (*Varanus salvator*), Pallid Ghost Crabs (*Ocypode pallidula*), and red ants (*Pheidologeton* spp.), or colonized by fungi (*Fusarium solani* species complex) due to decomposing leaves and organic material mixed within the sand nearest to the edge of forests (Mortia et al. 2008; Sarmiento-Ramírez et al. 2014; Staines and Booth 2019). This can lead to reduced embryonic survivorship, although we found that the hatching success rate was similar in both natural nests (83%) and relocated nests (82%) at Chagar Hutang beach. This may be attributed to the ongoing efforts by SEATRU staff to protect all nests, both natural and relocated from monitor lizard predation with a 1 × 1 m piece of plastic mesh secured over the nest site by a layer of sand, which prevents lizards from excavating nests. If it is considered desirable to increase male hatchling production, then the clutches that are rescued from areas of the beach subjected to water immersion should be relocated to areas of natural shade produced by trees fringing the beach, or to an artificial shade structure constructed close to the Sea Turtle Research Station at Chagar Hutang and managed by staff of SEATRU.

At the Tiga Ruang hatchery, it would be relatively easy to construct an artificial shade structure over the hatchery to reduce incubation temperatures and increase male hatchling production. Previous studies from within the region have indicated that 70–100% shade cloth was successful in maintaining nest temperatures between 28°–30° C, enough to produce male hatchlings at the Ma'Daerah hatchery site (van de Merwe et al. 2005), and that shaded hatcheries increased hatching success compared to open beach hatcheries in Setiu, Terengganu (Mutalib and Fadzly 2015). Jourdan and Fuentes (2015) warn that the impermeability of shade cloth prevented natural rainfall from cooling hatchery sand during their study, and therefore recommend the use of natural materials like palm fronds to build water-permeable shade structures. DoF staff (pers. comm.) that manages the hatchery in the Perhentian Islands believe that introducing artificial shade would only impede excess water evaporating from the sand after periods of heavy rain, increasing the prevalence of ant invasion and fungal infection within the hatchery. Hence, a good compromise would be the application of shade cloth or natural palm fronds along the perimeter of the hatchery fence, which would shade part of the hatchery in the afternoon and thus reduce sand temperature in the shaded area by 1°–2° C while also allowing for the evaporation of excess moisture from within the hatchery substrate. This could sufficiently reduce the incubation temperature of some nests and produce a 70% female to 30% male hatchling sex ratio across the entire hatchery (unpubl. data).

Our study found that hatching success was slightly lower (77%) at the Tiga Ruang hatchery compared to Chagar Hutang beach. This could be caused by several different factors, including ant and crab predation, fungal infection, or even the result of movement-induced mortality (Limpus et al. 1979), during the transportation of egg clutches from their natural nest site to the hatchery site. Hoh et al. (2020) report from their study of seven major nesting beaches in Peninsular Malaysia that fungal pathogens present at nesting beaches can become more prevalent within hatchery sites when the same sand is continuously reused annually for constructing artificial nests.

Feminized sex ratios and hatchling fitness.—A previous study conducted at Chagar Hutang Turtle Sanctuary in 1995 reported that mean TSP temperatures ranged from 27.9°–31.5° C, with nests incubated along the open beach producing 85% female hatchlings and nests shaded by natural vegetation producing 52% female hatchlings (Palaniappani et al. 2000). The most recent study, conducted 24 y later at the same rookery, reported considerably increased TSP temperatures ranging from 29.6°–32.5° C (Reboul et al. 2021). This

resulted in an increased female hatchling production: 100% females from unshaded open beach nests and 73% females in nests shaded by natural vegetation (Reboul et al. 2021). This, along with other studies (Hays et al. 2017; Jensen et al. 2018), provides evidence of the progressive feminization of sea turtle populations over time.

While warmer nest temperatures may at first result in an increase in mature females and thus an initial overall increase in hatchling production due to more eggs being laid, a shortage of males will eventually impact the overall fertilization rate of females in the population and result in a population decline (Jensen et al. 2018). Absent any adjustment of nest placement by females, Hays et al. (2017) suggest that sea turtle populations that already produce more than 80% female hatchlings will face the threat of extinction if global temperatures increase 2°–3° C within the next 50 y as predicted by the International Panel on Climate Change (Masson-Delmotte et al. 2018). Growing concern over the negative impacts global climate change may have on the incubation success and hatchling sex ratios of threatened sea turtle populations have justified the use of artificial cooling and shading strategies as important conservation efforts (Maulaney et al. 2012; Hill et al. 2015; Jourdan and Fuentes 2015; Mutalib and Fadzly 2015). Altering sex ratios by producing more males in hatcheries, however, could be detrimental for sea turtle conservation as fewer female hatchlings produced translate into fewer nesting turtles laying eggs in the future (Mrosovsky and Yntema 1980), reducing the population reproductive output and possibly leading to population declines (Tomillo et al. 2021). On the other hand, increasingly warmer environmental conditions could drive the number of males too low for all clutches to be fertilized, reducing hatchling production to critically low levels through decreased hatching and emergence success (Hays et al. 2017; Tomillo et al. 2021). More studies are needed to further document natural or functional reproductive sex ratios of mature turtles found at breeding sites, to better understand the impact that increased male hatchling production may have on the future population dynamics and reproductive success of nesting populations.

Future studies.—Although our study found hatchling sex ratios produced from relocated nests at both Chagar Hutang beach and Tiga Ruang hatchery to be highly female-biased, small sample size may have prevented us from establishing the true sex ratios of clutches incubated in hatcheries and naturally. Furthermore, the use of the middle third of incubation as a proxy for the TSP in this study is likely to have produced an underestimation of the average temperature experienced during the sex determination period (Girondot et al. 2018), and thus the sex ratio predicted

using this method is considered to under-represent the true female hatchling sex ratio. Ideal methods for determining the sex of sea turtle hatchlings should be non-invasive as well as inexpensive and easy to perform. Recently researchers have proposed analyzing relatively small samples of blood for the presence of sex steroid proteins known to play an essential role in the sex determination of embryos during development (Tezak et al. 2020). Although these methods are described as minimally invasive, they still require extracting blood from neonate hatchlings. The far less invasive method of testing amniotic fluid from laboratory incubated and hatched eggs have produced almost identical results as blood hormone assays (Gross et al. 1995; Xia et al. 2011). In addition, Kobayashi et al. (2015) reported sex steroid hormones were detectable in eggshell fragments recovered from turtle nests and successfully correlated to concentrations of sex hormones in blood samples taken from hatchlings of the same nest, indicating that the collection of eggshells might serve as an entirely non-invasive method for estimating the sex ratio of sea turtle hatchlings. The further pursuit and development of field techniques like these have the potential to be highly advantageous in their simplicity and non-invasive nature to better provide crucial physiological information, like the sex ratio of hatchlings in relation to their incubation temperature and nest environment.

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