



Genetic markers provide insight on origins of immature green turtles *Chelonia mydas* with biased sex ratios at foraging grounds in Sabah, Malaysia

Michael P. Jensen^{1,3}, Nicolas Pilcher^{2,*}, Nancy N. FitzSimmons^{1,4}

¹Institute for Applied Ecology, University of Canberra, ACT 2601, Australia

²Marine Research Foundation, 136 Lorong Pokok Seraya 2, 88540 Kota Kinabalu, Sabah, Malaysia

³Present address: Ocean Associates, under contract to the Marine Mammal and Turtle Division, Southwest Fisheries Science Center, National Marine Fisheries Service, NOAA, 8901 La Jolla Shores Dr., La Jolla, CA 92037, USA

⁴Present address: Australia Rivers Institute, Griffith University, Griffith, Qld 4222, Australia

ABSTRACT: An understanding of population dynamics is needed to assess the viability of migratory species. Monitoring of marine turtles at foraging grounds may detect changes in population trends that would take decades to be seen at nesting beaches. Mixed Stock Analysis using molecular markers provides a tool for estimating the origin of turtles sampled at foraging grounds. Here, we analysed mitochondrial DNA sequences of 90 immature green turtles at 2 foraging grounds in northwestern Sabah, Malaysia. We used data from 30 Indo-Pacific green turtle rookeries as the baseline for tracing the origin of turtles at the 2 foraging grounds. The inferred origins of turtles at the 2 locations were not different and indicated that the majority originated from 3 major populations in Southeast Asia, the Turtle Islands of Sarawak in northwestern Borneo (29%), the Turtle Islands Heritage Protected Area (TIHPA) (28%) and Peninsular Malaysia (25%). Previous analyses indicated a 1:4 female-biased sex ratio at the foraging grounds, and based on our results, this largely reflects the use of unshaded beach hatcheries at some of the source rookeries for decades, which resulted in mostly female hatchlings. This result is supported by differences in the origins of male and female turtles. The result suggests a greater proportion of males originating from Peninsular Malaysia and fewer males originating from Sarawak and possibly the TIHPA compared to females. We discuss the implications of hatchery practices that influence sex ratios of hatchlings and recommend future research to improve the management of marine turtles in the region.

KEY WORDS: Mixed stock analysis · mtDNA · Sex ratio · Female bias · Marine turtle · Conservation management

INTRODUCTION

Green turtles *Chelonia mydas* are circumglobally distributed and although they are the most abundant large herbivore in many shallow marine habitats, they are considered globally endangered (IUCN 2004). Numerous populations are at risk of extinction due to

a variety of anthropogenic threats (Wallace et al. 2011). Throughout Southeast Asia, regionally large rookeries (>2000 nests yr⁻¹) are found along the coasts of Indonesia and Peninsular Malaysia, the islands of eastern Malaysia (Sabah and Sarawak Turtle Islands) and the Tawi-Tawi Turtle Islands in the Philippines (Shanker & Pilcher 2003, Pilcher 2007),

*Corresponding author: npilcher@mrf-asia.org

with smaller rookeries (~100–500 nests yr⁻¹) scattered throughout (Pilcher 2007). Green turtle populations in Southeast Asia have long suffered severe impacts from fisheries, legal and illegal harvests of turtles and a large and widespread collection of eggs, causing major declines in many populations (Limpus et al. 2001, Shanker & Pilcher 2003, Seminoff 2004). While the majority of these populations remain depleted and continue to decline, one population in the Turtle Islands Heritage Protected Area (TIHPA) is showing signs of recovery as a result of long-term conservation efforts (Basintal 2002, Sabah Parks & Philippines Department of Environment and Natural Resources unpubl. data). The TIHPA includes 3 islands in Malaysia (Selingan, Bakkungan Kechil and Gulisaan) and 6 islands in Philippine waters (Boan, Langaan, Lihiman, Great Bakkungan, Taganak and Baguan), and together they are the largest nesting grounds for green turtles in Southeast Asia.

Within Southeast Asia, there is a less obvious threat affecting green turtle populations that is difficult, but important, to quantify. Starting as early as the 1950s in Sarawak and the 1960s in Terengganu and Sabah (Chan 2006), rookeries throughout Malaysia began relocating the majority of clutches laid and placing them in unshaded beach hatcheries for incubation (Leh et al. 1985, Tiwol & Cabanban 2000, San et al. 2004, Pilcher 2007). For many clutches, this meant taking them from where they had been laid under a dense tree canopy, thus considerably changing the nest temperature environment of many of the relocated clutches (Tiwol & Cabanban 2000, Salleh et al. 2012). Nests that experience sand temperatures above the pivotal temperature will produce higher proportions of female hatchlings because of temperature-dependent sex determination (Mrosovsky 1980, 1994). The relocation of eggs in Malaysia resulted in as much as a 100% female hatchling production from both the TIHPA (Chan 1988, Tiwol & Cabanban 2000) and hatcheries in Peninsular Malaysia (Limpus 1993, Ibrahim 1994), and this practice probably persisted for several decades. Given this, potentially millions of female hatchlings and only relatively few male hatchlings were released from these rookeries for a number of decades (Tiwol & Cabanban 2000, Shanker & Pilcher 2003, Pilcher 2010).

Several studies have stressed the importance of shading hatcheries to control nest temperatures (Standora & Spotilla 1985, van de Merwe et al. 2005, 2006), but partial shading only started in the late 1990s at rookeries in Southeast Asia. Since 1999, the main hatcheries in Peninsular Malaysia have been shading 70 to 100% of the hatchery, resulting in

more males being produced (van de Merwe et al. 2005). The Turtle Islands National Park in Sabah, Malaysia, shaded ~50% of the nests in hatcheries in the mid-1990s. However, the long-term release of predominantly female hatchlings is likely to have substantial impacts on population trends as the adult populations become increasingly female biased (Limpus et al. 2001).

While the higher breeding frequency of males (relative to females) may counteract female-biased sex ratios (Wright et al. 2012), there is concern that highly skewed sex ratios will eventually impact clutch fertility as males become a limited resource (Witt et al. 2010, Fuentes et al. 2011). This is particularly true when considering predictions of further female bias to hatchling recruitment caused by increasing temperatures in the future (Fuentes et al. 2010). While little is known about the degree of female bias a population can sustain, it has been suggested that concerns should be raised if male:female (M:F) sex ratios of hatchlings across all nesting beaches for a particular stock approach 1M:4F (i.e. 80% females) (Poloczanska et al. 2009). Depending on the population, green turtles do not reach sexual maturity until 20 to 50 yr after hatching (Chaloupka et al. 2004, Van Houtan et al. 2014). Therefore, the effects of increased sand temperature on hatchling sex ratios, whether from hatchery practices or climate change (Fuentes et al. 2010, Wallace et al. 2011), would not be detected at nesting beaches until decades later. Demographic research at foraging grounds is needed to monitor the recruitment of hatchling cohorts through time and better understand the impact of biased hatchling sex ratios. For example, in the Mediterranean, necropsy analyses of juvenile loggerhead turtles killed as bycatch in fisheries showed an overall sex ratio of approximately 1:1 (Casale et al. 2006), despite a female bias in hatchlings at many rookeries within the Mediterranean (e.g. Godley et al. 2001a,b, Fuller et al. 2013). It is hypothesised that at least some of the juvenile male turtles in the sample may have entered Mediterranean feeding grounds from rookeries in the Atlantic Ocean (Casale et al. 2006).

Several long-term studies using mark-recapture techniques on marine turtles at foraging grounds have shown the value of providing detailed sex- and size class-specific demographic information (Limpus & Chaloupka 1997, Chaloupka & Limpus 2001, 2002, Pilcher 2010), though little of this research has been done in Southeast Asia. In a recent foraging ground study in Malaysia, Pilcher (2010) found that the shallow waters surrounding the Mantanani Islands

(45 nautical miles northwest of Kota Kinabalu, Borneo) served as a developmental habitat for immature green turtles, with turtles recruiting at around 38 cm in curved carapace length (CCL), which would be around 5 to 10 yr old (Zug et al. 2002). Turtles larger than 62 cm CCL were generally absent from these areas, thus suggesting a residence period for immature turtles of 6 to 7 yr based on recruitment sizes and local growth rates (Pilcher 2010), which were similar to those of green turtles on the Great Barrier Reef (Chaloupka et al. 2004). Importantly, laparoscopic examinations similar to those performed by Miller & Limpus (2003) showed that this foraging aggregation had a highly female-biased sex ratio of 80% female, indicating a possible link with the biased sex ratios of hatchlings previously incubated at hatcheries in the region (e.g. Tiwol & Cabanban 2000) as well as natural sex bias observed at some beaches. It is not known, however, what proportion of the turtles at these foraging grounds originated from the different populations within the region.

Effective conservation management of marine turtle populations requires knowledge of the component rookeries that comprise regional breeding populations and the extent to which each of these populations contributes turtles to key foraging grounds in the region. Such information allows assessments of the overall level of risk faced by a population from threats at both nesting beaches and foraging grounds (Reece et al. 2006). For example, linking populations that may be affected by various anthropogenic activities, such as fisheries or subsistence harvest, hundreds or even thousands of kilometres away from foraging grounds (Bowen et al. 1995, Laurent et al. 1998, Boyle et al. 2009, Jensen et al. 2013a) will better inform conservation policy. Such insights have been gained from genetic studies using analyses of mitochondrial DNA (mtDNA) haplotype frequencies to define breeding populations (stocks) and mixed stock analysis (MSA) of turtles at foraging grounds to estimate the stock contributions. The number of studies using MSA has increased in the last decade, and these analyses are now an integral part of most marine turtle foraging ground studies (Jensen et al. 2013b).

In this study, we use mtDNA sequencing techniques to study immature green turtles at the Mantanani foraging site studied by Pilcher (2010) and some additional samples at a second site near Layang Layang Island, also off the northwestern coast of Borneo, Malaysia, to assess the natal origin of turtles using these foraging grounds. This study uses MSA to determine the origins of foraging male and female

juvenile green sea turtles to consider possible links with rookeries thought to have strongly female-biased offspring production in the recent past, including rookeries with a long tradition of relocating nests into unshaded hatcheries.

MATERIALS AND METHODS

Study site and sample collection

The 2 study sites at Mantanani (6° 71' N, 116° 35' E) and Layang Layang Island (7° 40' N, 113° 82' E) are located off the northwestern coast of Borneo in Sabah, Malaysia, and are separated from each other by approximately 290 km (Fig. 1). The foraging ground at Mantanani is approximately 25 km offshore of Sabah and straddles the main Sarawak migratory pathway used by green turtles and other marine organisms (Bali et al. 2000). In contrast, the foraging ground at Layang Layang lies approximately 280 km offshore and straddles several migratory routes for breeding green turtles (Liew et al. 1995, Chan & Liew 2001, Cheng 2002, World Wildlife Fund for Nature Indochina & N. Pilcher unpubl. data). Turtles were sampled around Mantanani (n = 94) in December 2006; January, April and October 2007; January, September and December 2008; and February 2009. These included 75 turtles reported on in Pilcher (2010). Sampling at Layang Layang (n = 12) was carried out only in October 2006. Immature green turtles were caught by rodeo-style capture in shallow water (Limpus & Reed 1985). All captured turtles were flipper tagged, weighed and measured for CCL (± 0.1 cm), and a skin biopsy was taken and stored in a 20% DMSO solution saturated with sodium chloride. Gender was determined for all first-time captures using laparoscopy procedures (Miller & Limpus 2003). Turtles were returned to the sea within 1 to 2 h of capture.

Characterisation of mtDNA haplotypes

DNA was extracted using the salting out method as described in Jensen et al. (2013a). DNA was re-suspended in LTE buffer (0.1 mM EDTA, 10 mM Tris, pH 7.5) and visualised for quality and quantity using 1% agarose gels. We amplified an ~800 bp fragment of the mtDNA control region using primers LTEi9 and H950 (Abreu-Grobois et al. 2006). PCRs were performed in 25 μ l reactions containing 1 \times reaction buffer, 1.5 mM MgCl₂, 0.25 mM of each

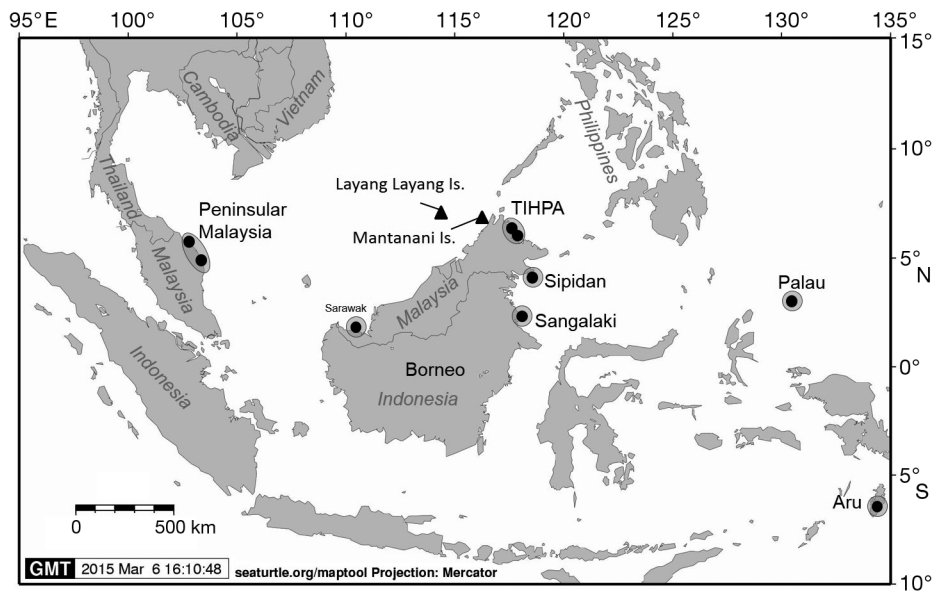


Fig. 1. Locations of the 7 most proximate genetic stocks for *Chelonia mydas* used as part of the 30 baseline sources for determining the origin of turtles at the 2 foraging sites studied off Mantanani and Layang Layang islands, Malaysia. Black dots represent individual rookeries, and gray circles group rookeries together into single stocks that are genetically undifferentiated. TIHPA: Turtle Islands Heritage Protected Area

dNTP, 10 μ M of each primer, 1.25 U of *Taq* polymerase and ~40 ng of template DNA. The PCR protocol consisted of a 5 min denaturing step (94°C) followed by 35 cycles of 45 s at 94°C (denaturing), 45 s at 52°C (annealing) and 45 s at 72°C (extension) and a final extension step of 5 min at 72°C. PCR products were purified using a polyethylene glycol cleanup procedure (Sambrook et al. 1989), and nucleotide sequences were determined for both strands of the PCR amplification products at the Macrogen (Korea) sequencing facility.

Clustal W was used to align all sequences as implemented in Geneious Pro 6.0.4 (Drummond et al. 2009). Haplotypes were identified by running a search against a collated database of known green turtle haplotypes. If no matching haplotypes were found, the BLAST procedure was used to search the GenBank database (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). Again, if no matching haplotypes were found, the sequence was identified as being unique and given a new haplotype name following the standard nomenclature for Indo-Pacific green turtles using the prefix CmP followed by the next available sequential number (P. Dutton pers. comm.). Following haplotype identification, a chi-square test was performed to determine if there were significant differences in haplotype frequencies between the 2 foraging grounds, between different years and between males and females.

Mixed stock analysis

The potential source baseline information for the MSA was assembled using mtDNA haplotype frequencies of 30 genetically differentiated green turtle breeding stocks identified across the Indo-Pacific (Dethmers et al. 2006, Cheng et al. 2008, Nishizawa et al. 2013, Dutton et al. 2014, Hamabata et al. 2014, Jensen et al. 2016). The inclusion of all 30 rookeries meant cropping sequences to 384 bp, which is the longest sequence used at some rookeries (e.g. rookeries in Taiwan and Japan). Results from this initial analysis ruled out possible contributions from distant rookeries for which only shorter sequence data were available (mean = 0% and upper 95% CI < 5; data not shown). This allowed us to perform a second analysis using only data from 25 rookeries that were most likely to contribute to the foraging grounds and for which longer 770 bp sequences were available (Table S1 in the Supplement at www.int-res.com/articles/suppl/n031p191_supp.xlsx).

A Bayesian approach using the program BAYES (Pella & Masuda 2001) was used to estimate the contribution of the baseline rookeries to the foraging grounds studied. This analysis was run using both uniform and weighted priors. In the analysis using uniform priors (Model 2), each of the rookeries was equally likely to contribute individuals to the foraging aggregations. For the weighted prior analysis

(Model 1), the potential contributions of different rookeries to the foraging aggregations were weighted relative to the size of the rookery. Thus, when the program could not distinguish between 2 genetically similar rookeries, the larger rookery was assumed to contribute more individuals to the foraging aggregation. We ran the MSA 3 times for each model, including (1) all samples combined, (2) all females from the 2 foraging areas, and (3) all males for the 2 foraging areas. Ten chains of 50 000 Markov chain Monte Carlo steps were run, each with different starting points. A burn-in of 25 000 runs was used before calculating the posterior distribution. The Gelman and Rubin shrink factor diagnostic was computed to test that all chains had converged (Pella & Masuda 2001).

RESULTS

A total of 106 juvenile green turtle samples were obtained from the foraging grounds in Malaysia. Samples from Mantanani Island included 23 females and 3 males from 2006, 24 females and 9 males from 2007, 11 females and 3 males from 2008 and 17 females and 4 males from 2009. An additional 12 samples were obtained from Layang Layang Island (9 females and 3 males) in 2006. The sex ratio for all turtles caught was biased, but the difference in sex ratios was not significantly different at the 2 sites ($p = 0.71$, $n = 106$, Fisher exact test) and ranged from 1M:3F ($n = 12$) at Layang Layang Island to 1M:4F ($n = 94$) at Mantanani.

A total of 90 juvenile green turtle samples were successfully amplified by PCR (Table 1). From these, 16 haplotypes were identified from the 770 bp mtDNA control region fragment, of which 4 (CmpP49.5, CmpP154.1, CmpP215.1 and CmpP187.1) were previously undescribed (GenBank ID KM923921, KM923922, KM923923, KM923924). These were each found in only 1 to 3 individuals and together comprised 10% of all sampled individuals. Although these 4 orphan haplotypes were found in a larger proportion of male (22%) than female (6%) samples, the difference was not significant ($p = 0.058$, $n = 90$, Fisher exact test). The most common haplotypes were CmpP49.1, CmpP87.1, CmpP57.1 and CmpP91.1, which were observed in 11 to 22% of the total sample (Table 1).

There was no evidence for temporal variation in haplotype composition between turtles sampled in different years at Mantanani (2006 versus 2007 [$\chi^2 = 15.03$, $p = 0.30$], 2006 versus 2008 [$\chi^2 = 13.71$, $p = 0.24$] or 2007 versus 2008 [$\chi^2 = 15.09$, $p = 0.23$]) or between Layang Layang (2006) and Mantanani in different years (2006 [$\chi^2 = 6.69$, $p = 0.75$], 2007 [$\chi^2 = 13.87$, $p = 0.30$] and 2008 [$\chi^2 = 8.97$, $p = 0.53$]). When sampling years were combined, the 2 foraging areas continued to display genetic homogeneity ($\chi^2 = 14.13$, $p = 0.516$). Additionally, there was no significant difference in haplotype frequencies between males and females based on samples combined from both foraging areas ($\chi^2 = 17.81$, $p = 0.21$), although this was based on a small sample size for male turtles ($n = 19$).

Table 1. Frequencies of 16 mitochondrial DNA D-loop haplotypes (770 bp) found in juvenile *Chelonia mydas* foraging near Mantanani and Layang Layang islands, Malaysia. Data are shown by sampling year and sex across the 2 foraging areas. Shaded columns show orphan haplotypes not found at any rookery to date

	Total	CmpP19.1	CmpP20.1	CmpP40.1	CmpP49.1	CmpP49.3	CmpP57.1	CmpP57.2	CmpP82.1	CmpP87.1	CmpP91.1	CmpP98.1	CmpP104.1	Orphan haplotype			
														CmpP49.5	CmpP154.1	CmpP187.1	CmpP215.1
Mantanani total	78	4	4	2	19	3	11	4	1	13	7	0	3	2	3	1	1
2006	26	0	1	0	10	0	4	0	0	5	4	0	1	0	0	1	0
2007	33	3	2	1	5	0	6	4	1	6	2	0	1	0	2	0	0
2008	10	1	1	0	2	1	1	0	0	1	0	0	1	2	0	0	0
2009	9	0	0	1	2	2	0	0	0	1	1	0	0	0	1	0	1
Layang Layang total	12	1	1	0	1	0	1	1	0	2	3	1	0	1	0	0	0
2007	12	1	1	0	1	0	1	1	0	2	3	1	0	1	0	0	0
Mantanani and Layang Layang combined																	
Male	19	0	1	0	4	0	2	1	0	3	2	1	1	1	2	0	1
Female	71	5	4	2	16	3	10	4	1	12	8	0	2	2	1	1	0

Mixed stock analysis

The overall MSA using uniform priors (Model 2) indicated that several stocks were potential contributors to the foraging grounds (having upper 95% CIs larger than zero), but only 5 stocks had estimated mean contributions >5% (Table S1 in the Supplement). Nonetheless, the CIs for all estimates were large, and the lower 2.5% CI included zero in most cases. This likely reflects the presence of a number of haplotypes in the region that are broadly distributed among rookeries that also occur at high frequencies. Thus, although this generates some uncertainty surrounding individual source contribution estimates, the results are robust in showing that most (>80%) of the juvenile turtles originate from genetic stocks previously identified as the Sarawak Management Unit (MU), the Peninsular Malaysia MU and the Sulu Sea MU, which includes the TIHPA (Dethmers et al. 2006). Overall, the MSA results from both the uniform and the weighted prior analyses showed similar results, with only minor differences (Table S1). However, the unweighted MSA estimates for contributions from the southeastern Sabah MU (i.e. Sipadan) were disproportionately large relative to those from the TIHPA, given the very small population size of the southeastern Sabah MU in comparison to that of the TIHPA population. When rookery size was

accounted for through the weighted prior (Model 1), the estimated contribution from the southeastern Sabah MU decreased relative to that from the TIHPA (Table S1). For this reason, the results from Model 1 (using population priors) are presented in the 'Discussion'. When running the MSA by gender, the estimated mean contributions from the Turtle Islands of Sarawak and the TIHPA were less for males (2 and 21%, respectively) than for females (25 and 30%, respectively), with larger contributions from Peninsular Malaysia for males (62%) versus females (30%) as well as other rookeries (Fig. 2). These results, however, need to be taken with caution, as the estimates were surrounded by large CIs and were thus not significantly different.

DISCUSSION

Genetic analyses of green turtle aggregations at the 2 Malaysian foraging grounds showed these to be genetically undifferentiated and that the sites are used by turtles from multiple regional rookeries. The majority of turtles appear to originate from 3 large regional rookeries at Sarawak in northwestern Borneo (29%), the TIHPA off the northeastern coast of Borneo (28%) and the eastern coast of Peninsular Malaysia (25%). Small contributions were also estimated from Aru (8%), Indonesia, as well as potential contributions from Ashmore Reef, North West Cape and northern Great Barrier Reef (nGBR) populations in Australia. The 5 turtles with haplotype Cmp19.1 are likely to originate from an unknown or unsampled rookery. A shorter 384 bp fragment of this haplotype (Cmp19) has been found in turtles nesting in Taiwan but at a very low frequency (4%) (Cheng et al. 2008), but given that the most frequent haplotype (Cmp18 = 65%) found in Taiwanese rookeries was not found among the Malaysian foraging assemblage, the Taiwanese rookeries remain an unlikely source. An individual with a haplotype (Cmp98.1) only found to date at nGBR rookeries provided an unanticipated, novel insight of potential long-distance contributions. It may indicate that a small proportion of turtles have originated from nesting

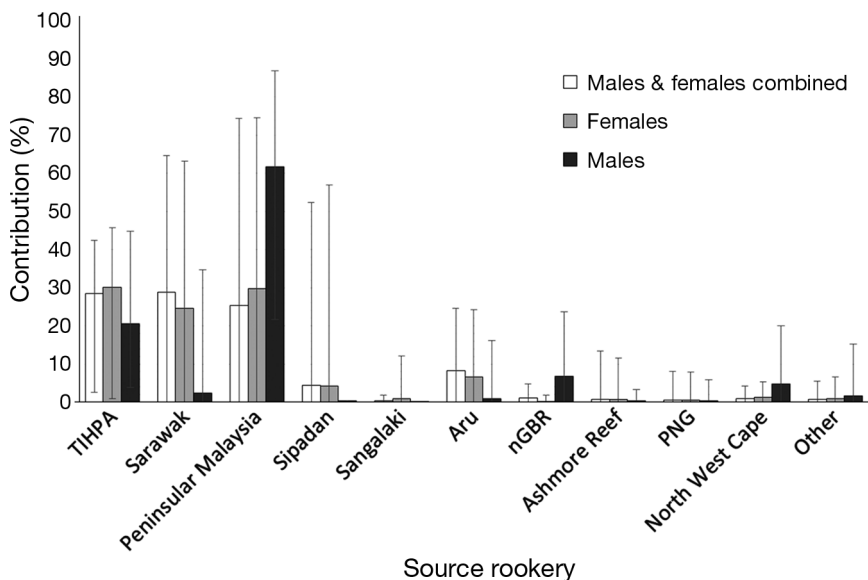


Fig. 2. Contributions from regional green turtle genetic stocks to foraging aggregations around Mantanani and Layang Layang islands, Malaysia, for all turtles combined (white) as well as females (light gray) and males (dark gray) separately. Error bars show the 95% CI. TIHPA: Turtle Islands Heritage Protected Area; nGBR: northern Great Barrier Reef; PNG: Papua New Guinea

beaches more than 3800 km to the southeast. Similar long-distance migrations have been observed in a few green turtles flipper tagged while nesting in the nGBR that were found using foraging grounds in eastern Indonesia 2770 km from their nesting beach (Limpus et al. 2003). This suggests that oceanic currents might carry migrating post-hatchlings north and westwards from the nGBR into waters of the Banda and Celebes seas and that some eventually take up residence there, resulting in concomitant long-distant migrations by adults to natal breeding regions.

Being able to assess population structure and demographic parameters across all age classes and both sexes is important for accurate population assessments. The green turtles sampled at the 2 foraging grounds were all immature turtles that presumably use these areas as developmental habitat, given the lack of adult turtles observed at the foraging area. While a few adult turtles have been observed in the deeper water surrounding Mantanani, data suggest this foraging area is little used by larger turtles (Pilcher 2010). It is not uncommon for green turtles to select developmental habitats that are only used by immature turtles. In the Bahamas, green turtles recruit into juvenile developmental habitats and stay for several years. When they reach approximately 65 cm CCL, they shift to other foraging grounds throughout the Caribbean (Bjorndal et al. 2003). This contrasts with many foraging grounds on the Great Barrier Reef, where both immature and adult turtles are found foraging on the same reef (Limpus & Reed 1985, Jensen et al. 2016). Additionally, several individuals have transitioned from juvenile through to adult stages at the same location (Limpus & Chaloupka 1997). It is unknown where green turtles foraging in northwestern Sabah go once they reach ~60 cm CCL, but they likely take up residence in nearby foraging habitats suitable for adult turtles, possibly to the north along the Balabac Straits between Borneo and the Philippines, a known hotspot for adult foraging green turtles (e.g. Pilcher 2012).

The 2 foraging aggregations studied here, located 290 km apart, did not differ in the sex ratio of juvenile turtles or in haplotype frequencies. If the Sarawak and TIHPA rookeries are indeed 2 of the main sources of recruitment into these foraging aggregations, and they have been producing almost entirely female hatchlings as has been suggested, then the observed higher female than male contribution from these populations would be consistent with expectations of highly female-biased hatchling

production at the Sabah and Philippine turtle islands, not only at hatcheries but also in nests left *in situ* at some beaches (Trono 1991). For the rookeries in Sarawak, it has been estimated that hatchery-raised hatchlings were also highly female biased (80–96% females) in comparison to nests left *in situ* (San et al. 2004). While unshaded hatchery practices at several rookeries in Peninsular Malaysia were also thought to produce mainly female hatchlings from 1961 to 1993 (Limpus 1993, Ibrahim 1994), the shading of hatcheries in 1999 reduced nest temperatures enough to produce more males (van de Merwe et al. 2005). One study from the hatchery in Ma'Daerah in Peninsular Malaysia even indicated a male-biased hatchling production averaging 72% males (van de Merwe et al. 2005). In addition, natural sex ratios from the largest rookery in Peninsular Malaysia, Pulau Redang, were female biased in open parts of the beach (85.4% females) but were almost equal for nests laid in the shaded part of the beach (52.9% females) (Palaniappan et al. 2000). Considering that studies from Peninsular Malaysia have shown that 60% of green turtles have a preference for nesting under trees (Salleh et al. 2012), this suggests that at least some key rookeries along the eastern coast of Peninsular Malaysia are producing males. The greater proportion of male than female juvenile turtles at our study sites originating from Peninsular Malaysia supports these expectations, although our results are based on a sample size of only 19 males.

Furthermore, the observation that there may be a higher proportion of males with orphan haplotypes (21%) relative to females (6%) suggests that male turtles may have originated from as-yet-unsampled rookeries. However, further sampling is needed to test this hypothesis. Also, the turtle with haplotype Cmp98.1, which has only been found in the nGBR rookeries, was a male turtle. These results are similar to scenarios for juvenile loggerheads in the Mediterranean, where mixed foraging aggregations were comprised of females mostly originating from local rookeries, while males are hypothesised to mostly originate from more distant male-producing rookeries outside the Mediterranean (Casale et al. 2006).

Female bias in juvenile green turtle aggregations is not uncommon and has been reported from across the globe. Highly female-biased sex ratios similar to our study have been reported from San Diego Bay, USA (1M:3.5F) (Allen et al. 2015) and Clack Reef, Australia (1M:4.2F) (Limpus et al. 2009). Moderately female-biased populations of ~1M:2F were observed at Moreton Bay (Limpus et al. 1994) and Shoalwater

Bay (Limpus et al. 2005), Australia, as well as in New Zealand (Godoy et al. 2016) and Qatar (Pilcher et al. 2015), while equal sex ratios have been reported in Hawaii (Wibbels et al. 1993). However, the recruitment patterns of juvenile green turtle are often complex, and the sex ratios at mixed foraging aggregations are the result of sex ratios produced at multiple rookeries over several years.

Being able to determine the sex of turtles at feeding grounds and link turtles to regional rookeries is important to improve our understanding of population dynamics. In addition, a better knowledge of the variability of sex ratios at regional rookeries (Fuller et al. 2013), whether natural or artificial, is needed to fully understand the patterns observed at the 2 foraging areas. Also, an increased sample size (>60 individuals) for both males and females is needed to provide more robust analyses. Nonetheless, given the high female bias and the MSA estimate that the majority of turtles at the 2 foraging grounds likely originate from the main hatcheries at Sarawak and the TIHPA, it is clear that the problem of sex-biased hatchling production, whether natural or artificial, needs to be addressed.

Use of temperature probes at rookeries throughout the area can be used to gauge sex ratios of hatchlings based on an estimated pivotal temperature of $29.4 \pm 0.6^\circ\text{C}$ for green turtles in eastern Peninsular Malaysia (Whittier et al. 2003). Use of hatcheries throughout the region remains an important conservation action to protect against egg poaching, flooding, predation (such as by varanid lizards) and destruction by root invasion (Salleh et al. 2012), and this can be used to advantage to adjust sex ratios into the future. In recent years, shading of hatcheries has become common practice at most rookeries, and it will be important to continue monitoring changes to the sex ratio and the composition of juvenile turtles at these feeding grounds.

When interpreting the results of MSA, consideration must be given to whether all rookeries in the region have been genetically characterized or if the sample size from any given rookery may be too small to give an accurate representation of haplotype frequencies, especially of rare haplotypes. These shortcomings apply to this study as evidenced by the proportion of samples (10%) with haplotypes not identified at any rookery. Although the baseline sample covers the majority of the main nesting populations in the region, there are gaps in the coverage, especially from the region north of Borneo. Many small to medium rookeries have been recorded for areas including Vietnam (Hamann et al. 2006), Tai-

wan (Cheng et al. 2009) and China (Shizheng & Shi 2009), but genetic data are only available for Taiwan (Cheng et al. 2009). Recent satellite tagging studies indicate that the northwestern Sabah adult foraging grounds are composed of turtles coming from numerous rookeries for which there are no genetic data including northwestern Sabah, South China Sea, Vietnam (N. J. Pilcher pers. obs.), and Terengganu (van de Merwe et al. 2009). A more comprehensive genetic sampling of rookeries is needed for the region and should be a priority for future work. Furthermore, widely shared haplotypes at dominant frequencies may reduce the power of the MSA. This is observed in locations with haplotype Cmp49.1, which is common and widespread in Southeast Asia, as well as haplotypes Cmp57.2, Cmp40.1 and Cmp91.1 (Jensen et al. 2016). These shared haplotypes are contributors to the large uncertainty surrounding the estimates for these populations. Whole mitogenome sequencing and microsatellites found in the mtDNA control region have added resolution to green turtle rookeries in both the Mediterranean and the Caribbean (Shamblin et al. 2012, Tikochinski et al. 2012) and may offer a better way to distinguish common and shared haplotypes in Southeast Asia in the future.

Insights provided by this study emphasise the need for long-term management plans that address historical impacts as well as contemporary ones and the importance of foraging ground studies to investigate the viability of turtle populations. There is a need for management practices to adapt so they reflect the biological requirements of the turtles themselves, particularly as these relate to temperature. In hatchery operations, factors such as nest depth and shading can be manipulated to influence nest temperature and thereby hatchling sex (van de Merwe et al. 2005, 2006). Effective conservation action at nesting beaches is extremely important for marine turtle conservation and recovery, as is the protection of turtles and habitat at regional foraging areas, and what happens in one area may directly impact the other. Understanding the factors that drive the foraging ground demographic structure and composition allows for a forward-looking model to predict trends. This research provides initial data towards a better understanding of connectivity between nesting and foraging habitats for green turtles in Southeast Asia. It also highlights the strengths of combining genetic data to estimate the origin of turtles at foraging grounds with information on the specimens' sex and the limitations that need to be addressed in future work.

Acknowledgements. We are grateful to D. Pilcher, F. Pilcher, C. Pilcher and M. Ruf for their assistance in turtle rodeo captures. This project was funded by the ExxonMobil Foundation, the Western Pacific Regional Fisheries Management Council and the Department of Conservation, Forestry Bureau, Taiwan Republic of China. Thanks to the University of Canberra and the Institute for Applied Ecology for support of the laboratory analyses. Also, thanks to C. Allen and K. Stewart for valuable comments on the manuscript. All financial support is gratefully acknowledged. This work was carried out under Malaysian EPU Research Permit 1551.

LITERATURE CITED

- Abreu-Grobois FA, Horrocks JA, Formia A, Dutton PH and others (2006) New mtDNA D-loop primers which work for a variety of marine turtle species may increase the resolution of mixed stock analysis. In: Frick M, Panagopoulou A, Rees AF, Williams K (compilers) Book of abstracts. Proc 26th Annu Symp Sea Turtle Biol Conserv. International Sea Turtle Society, Athens, p 179
- Allen CD, Robbins MN, Eguchi T, Owens DW and others (2015) First assessment of the sex ratio for an East Pacific green sea turtle foraging aggregation: validation and application of a testosterone ELISA. PLOS ONE 10: e0138861
- Bali J, Liew HC, Chan EH, Tisen OB (2000) Long distance migration of green turtles from the Sarawak Turtle Islands, Malaysia. In: Mosier A, Foley A, Brost B (compilers) Proc 20th Annu Symp Sea Turtle Biol Conserv. NOAA Tech Memo NMFS-SEFSC-477, p 32–33
- Basintal P (2002) Sea turtle conservation at the Sabah's Turtle Islands Park, Malaysia. In: Kinan I (ed) Proc West Pac Sea Turtle Coop Res Manage Workshop, Honolulu, HI, 5–8 February 2002. Western Pacific Regional Fishery Management Council, Honolulu, HI, p 151–161
- Bjorndal KA, Bolten AB, Chaloupka MY (2003) Survival probability estimates for immature green turtles *Chelonia mydas* in the Bahamas. Mar Ecol Prog Ser 252:273–281
- Bowen BW, Abreu-Grobois FA, Balazs GH, Kamezaki NI, Limpus CJ, Ferl RJ (1995) Trans-Pacific migrations of the loggerhead turtle (*Caretta caretta*) demonstrated with mitochondrial DNA markers. Proc Natl Acad Sci USA 92: 3731–3734
- Boyle MC, FitzSimmons NN, Limpus CJ, Kelez S, Velez-Zuazo X, Waycott M (2009) Evidence for transoceanic migrations by loggerhead sea turtles in the southern Pacific Ocean. Proc R Soc B 276:1993–1999
- Casale P, Lazar B, Pont S, Tomás J and others (2006) Sex ratios of juvenile loggerhead sea turtles *Caretta caretta* in the Mediterranean Sea. Mar Ecol Prog Ser 324:281–285
- Chaloupka M, Limpus C (2001) Trends in the abundance of sea turtles resident in southern Great Barrier Reef waters. Biol Conserv 102:235–249
- Chaloupka M, Limpus C (2002) Survival probability estimates for the endangered loggerhead sea turtle resident in southern Great Barrier Reef waters. Mar Biol 140: 267–277
- Chaloupka M, Limpus C, Miller J (2004) Green turtle somatic growth dynamics in a spatially disjunct Great Barrier Reef metapopulation. Coral Reefs 23:325–335
- Chan J 1988. Sex identification of green sea turtle (*Chelonia mydas*) hatchlings using histological techniques. DVM thesis, University of the Philippines, Quezon City
- Chan EH (2006) Marine turtles in Malaysia: on the verge of extinction? Aquat Ecosyst Health Manage 9:175–184
- Chan EH, Liew HC (2001) Sea turtles. In: Ong JE, Gong WK (eds) The encyclopedia of Malaysia, Vol 6: the seas. Archipelago Press, Singapore, p 74–75
- Cheng IJ (2002) Current sea turtle research and conservation in Taiwan. In: Kinan I (ed) Proc West Pac Sea Turtle Coop Res Manage Workshop, Honolulu, 5–8 February, 2002. Western Pacific Regional Fishery Management Council, Honolulu, HI, p 185–191
- Cheng IJ, Dutton PH, Chen CL, Chen HC, Chen YH, Shea JW (2008) Comparison of the genetics and nesting ecology of two green turtle rookeries. J Zoo Lond 276: 375–384
- Cheng IJ, Huang CTT, Hung PYY, Ke BZ, Kuo CWW, Fong CLL (2009) Ten years of monitoring the nesting ecology of the green turtle, *Chelonia mydas*, on Lanyu (Orchid Island), Taiwan. Zool Stud 48:83–94
- Dethmers KEM, Broderick D, Moritz C, FitzSimmons NN and others (2006) The genetic structure of Australasian green turtles (*Chelonia mydas*): exploring the geographical scale of genetic exchange. Mol Ecol 15:3931–3946
- Drummond AJ, Ashton B, Cheung M, Heled J and others (2009) Geneious pro v4.7. www.geneious.com
- Dutton PH, Jensen MP, Frutchey K, Frey A and others (2014) Genetic stock structure of green turtle (*Chelonia mydas*) nesting populations across the Pacific Islands. Pac Sci 68: 451–464
- Fuentes MMPB, Hamann M, Limpus CJ (2010) Past current and future thermal profiles for green turtle nesting grounds: implications from climate change. J Exp Mar Biol Ecol 383:56–64
- Fuentes MMPB, Limpus CJ, Hamann M (2011) Vulnerability of sea turtle nesting grounds to climate change. Glob Change Biol 17:140–153
- Fuller WJ, Godley BJ, Hodgson DJ, Reece SE, Witt MJ, Broderick AC (2013) Importance of spatio-temporal data for predicting the effects of climate change on marine turtle sex ratios. Mar Ecol Prog Ser 488:267–274
- Godley BJ, Broderick AC, Mrosovsky N (2001a) Estimating hatchling sex ratios of loggerhead turtles in Cyprus from incubation durations. Mar Ecol Prog Ser 210:195–201
- Godley BJ, Broderick AC, Downie JR, Glen F and others (2001b) Thermal conditions in nests of loggerhead turtles: further evidence suggesting female skewed sex ratios of hatchling production in the Mediterranean. J Exp Mar Biol Ecol 263:45–63
- Godoy DA, Smith ANH, Limpus C, Stockin KA (2016) The spatio-temporal distribution and population structure of green turtles (*Chelonia mydas*) in New Zealand. N Z J Mar Freshw Res doi:10.1080/00288330.2016.1182034
- Hamabata T, Kamezaki N, Hikida T (2014) Genetic structure of green turtle (*Chelonia mydas*) peripheral populations nesting in the northwestern Pacific rookeries: evidence for northern refugia and postglacial colonization. Mar Biol 161:495–507
- Hamann M, Cuong CT, Hong ND, Thuoc P, Thuhien BT (2006) Distribution and abundance of marine turtles in the Socialist Republic of Viet Nam. Biodivers Conserv 15: 3703–3720
- Ibrahim K (1994) The status of marine turtle conservation in Peninsular Malaysia. In: Nacu A, Trono R, Palma JA, Torres D, Agas F Jr (eds) Proceedings of the First ASEAN Symposium – Workshop on marine turtle conservation, Manila, p 87–103

- IUCN (2004) The IUCN Redlist. www.iucnredlist.org (accessed 8 Oct 2016)
- Jensen MP, FitzSimmons NN, Dutton PH, Michael P (2013a) Molecular genetics of sea turtles. In: Wyneken J, Lohmann KJ, Musick JA (eds) *The biology of sea turtles*, Vol 3. CRC Press, Boca Raton, FL, p 135–154
- Jensen MP, Limpus CJ, Whiting SD, Guinea M and others (2013b) Defining olive ridley turtle *Lepidochelys olivacea* management units in Australia and assessing the potential impact of mortality in ghost nets. *Endang Species Res* 21:241–253
- Jensen MP, Bell I, Limpus CJ, Hamann M and others (2016) Spatial and temporal genetic variation among size classes of green turtles (*Chelonia mydas*) provides information on oceanic dispersal and population dynamics. *Mar Ecol Prog Ser* 543:241–256
- Laurent L, Casale P, Bradai MN, Godley BJ and others (1998) Molecular resolution of marine turtle stock composition in fishery bycatch: a case study in the Mediterranean. *Mol Ecol* 7:1529–1542
- Leh CMU, Poon SK, Siew YC (1985) Temperature-related phenomena affecting the sex of green turtle (*Chelonia mydas*) hatchlings in the Sarawak Turtle Islands. *Sarawak Mus J* 55:183–193
- Liew HC, Chan EH, Papi F, Luschi P (1995) Long distance migration of green turtles from Redang Island, Malaysia: the need for regional cooperation in sea turtle conservation. In: Devaux B (ed) *Proc Int Congr Chelonian Conserv, Gonfaron*, 6–10 July 1995. SOPTOM, Gonfaron, p 73–75
- Limpus CJ (1993) Recommendations for conservation of marine turtles in Peninsula Malaysia. Report to Department of Fisheries, Ministry of Agriculture, Malaysia. Queensland Department of Environment and Heritage, Brisbane
- Limpus C, Chaloupka M (1997) Nonparametric regression modelling of green sea turtle growth rates (southern Great Barrier Reef). *Mar Ecol Prog Ser* 149:23–34
- Limpus CJ, Reed PC (1985) The green turtle, *Chelonia mydas*, in Queensland: a preliminary description of the population structure in a coral reef feeding ground. In: Grigg G, Shine R, Ehmann H (eds) *Biology of Australasian frogs and reptiles*. Royal Zoological Society of New South Wales, Mosman, p 47–52
- Limpus CJ, Couper PJ, Read MA (1994) The green turtle, *Chelonia mydas*, in Queensland: population structure in a warm temperate feeding area. *Mem Queensl Mus* 35:139–154
- Limpus CJ, Al-Ghais SMM, Mortimer JA, Pilcher NJ (2001) Marine turtles in the Indian Ocean and Southeast Asian region: breeding, distribution, migration and population trends. *Convention on Migratory Species*, Manila
- Limpus CJ, Miller JD, Parmenter CJ, Limpus DJ (2003) The green turtle, *Chelonia mydas*, population of Raine Island and the northern Great Barrier Reef: 1843–2001. *Mem Queensl Mus* 49:349–440
- Limpus CJ, Limpus DJ, Arthur KE, Parmenter CJ (2005) Monitoring green turtle population dynamics in Shoalwater Bay: 2000–2004. Res Pub No. 83, Great Barrier Reef Marine Park Authority, Townsville
- Limpus CJ, Bell IP, Miller JD (2009) Mixed stocks of green turtles foraging on Clack Reef, northern Great Barrier Reef identified from long term tagging studies. *Mar Turtle Newsl* 123:3–5
- Miller JD, Limpus CJ (2003) Torres Strait marine turtle resources. In: Lawrence D, Cansfield-Smith T (eds) *Torres Strait Baseline Study Conference*. CRC Press, Boca Raton, FL, p 213–226
- Mrosovsky N (1980) Thermal biology of sea turtles. *Am Zool* 20:531–547
- Mrosovsky N (1994) Sex ratios of sea turtles. *J Exp Zool* 270:16–27
- Nishizawa H, Naito Y, Suganuma H, Abe O and others (2013) Composition of green turtle feeding aggregations along the Japanese archipelago: implications for changes in composition with current flow. *Mar Biol* 160:2671–2685
- Palaniappan PM, Heng CE, Chark LH (2000) Spatial and temporal changes in sex ratios of green turtle (*Chelonia mydas*) hatchlings in Pulau Redang, Malaysia. In: Kalb H, Wibbels T (compilers) *Proc 19th Annu Symp Sea Turtle Biol Conserv*. NOAA Tech Memo NMFS-SEFSC-443, p 129–130
- Pella J, Masuda M (2001) Bayesian methods for analysis of stock mixtures from genetic characters. *Fish Bull* 99:151–167
- Pilcher NJ (2007) Regional action plan for the conservation of marine turtles and their habitats in the Sulu-Sulawesi seascape. Conservation International Philippines. www.conservation.org/archive/philippines/publications/Documents/Marine_Turtle_Regional_Action_Plan.pdf (accessed 15 Oct 2016)
- Pilcher NJ (2010) Population structure and growth of immature green turtles at Mantanani, Sabah, Malaysia. *J Herpetol* 44:168–171
- Pilcher NJ (2012) Aerial surveys of leatherback nesting and foraging habitat in the Sulu-Sulawesi region, off Borneo, Malaysia. Project NA09NMF4540275 Final Report. NMFS Pacific Islands Regional Office, Honolulu, HI
- Pilcher NJ, Al-Maslamani I, Williams J, Gasang R, Chikhi A (2015) Population structure of marine turtles in coastal waters of Qatar. *Endang Species Res* 28:163–174
- Poloczanska ES, Limpus CJ, Hays GC (2009) Vulnerability of marine turtles to climate change. In: Sims DW (ed) *Advances in marine biology*. Academic Press, Burlington, MA, p 151–211
- Reece JS, Ehrhart LM, Parkinson CL (2006) Mixed stock analysis of juvenile loggerheads (*Caretta caretta*) in Indian River Lagoon, Florida: implications for conservation planning. *Conserv Genet* 7:345–352
- Salleh SM, Yobe M, Sah SA (2012) The distribution and conservation status of green turtles (*Chelonia mydas*) and olive ridley turtles (*Lepidochelys olivacea*) on Pulau Pinang beaches (Malaysia), 1995–2009. *Trop Life Sci Res* 23:63–76
- Sambrook J, Fritsch EF, Maniatis T (1989) *Molecular cloning: a laboratory manual*, Vol 3. Cold Spring Harbour Laboratory Press, New York, NY
- San CS, Liew HC, Chan EH (2004) Sex ratios of green turtle hatchlings (*Chelonia mydas*) of Sarawak Turtle Island, Sarawak, Malaysia. In: Coyne MS, Clarck RD (compilers) *Proc 21st Annu Symp Sea Turtle Biol Conserv*. NOAA Tech Memo NMFS-SEFSC-528, p 309–310
- Seminoff JA (2004) *Chelonia mydas*. The IUCN Red List of Threatened Species 2004: e.T4615A11037468. <http://dx.doi.org/10.2305/IUCN.UK.2004.RLTS.T4615A11037468.en>. (downloaded 7 Oct 2016)
- Shamblin BM, Bjordal KA, Bolten AB, Hillis-Starr ZM, Lundgren I, Naro-Maciel E, Nairn CJ (2012) Mitogenomic sequences better resolve stock structure of southern greater Caribbean green turtle rookeries. *Mol Ecol* 21:2330–2340

- Shanker K, Pilcher NJ (2003) Marine turtle conservation in South and Southeast Asia: hopeless cause or cause for hope? *Mar Turtle Newsl* 100:43–51
- Shizheng W, Shi H (2009) Sea turtle conservation status in Paracel Islands and case analysis of sea turtle conservation in Hainan Province. In: Chan EH, Pilcher N, Hiew K (eds) Report of the workshop on regional cooperation to address direct capture of sea turtles: 1–3 June 2009, Kuala Terengganu, Malaysia. Universiti Malaysia Terengganu Press, Kuala Terengganu, p 16–17
- ▶ Stadora EA, Spotilla JR (1985) Temperature dependent sex determination in sea turtles. *Copeia* 1985:711–722
- ▶ Tikochinski Y, Bendelac R, Barash A, Daya A, Levy Y, Friedmann A (2012) Mitochondrial DNA STR analysis as a tool for studying the green sea turtle (*Chelonia mydas*) populations: the Mediterranean Sea case study. *Mar Genomics* 6:17–24
- Tiwol CW, Cabanban AS (2000) All female hatchlings from the open-beach hatchery at Gulisaan Island, Turtle Islands Park, Sabah. In: Pilcher NJ, Ismail MG (eds) Sea turtles of the Indo-Pacific: research management and conservation. Asean Academic Press, Kuala Lumpur, p 218–227
- Trono RB (1991) Philippine marine turtle conservation program. *Mar Turtle Newsl* 53:5–7
- ▶ van de Merwe JP, Ibrahim K, Whittier JM (2005) Effects of hatchery shading and nest depth on the development and quality of *Chelonia mydas* hatchlings: implications for hatchery management in Peninsular Malaysia. *Aust J Zool* 53:205–211
- ▶ van de Merwe JP, Ibrahim K, Whittier JM (2006) Effects of nest depth, shading, and metabolic heating on nest temperatures in sea turtle hatcheries. *Chelonian Conserv Biol* 5:210–215
- ▶ van de Merwe JP, Ibrahim K, Lee SY, Whittier JM (2009) Habitat use by green turtles (*Chelonia mydas*) nesting in Peninsular Malaysia: local and regional conservation implications. *Wildl Res* 36:637–645
- ▶ Van Houtan KS, Hargrove SK, Balazs GH (2014) Modeling sea turtle maturity age from partial life history records. *Pac Sci* 68:465–477
- Wallace BP, DiMatteo AD, Bolten AB, Chaloupka MY and others (2011) Global conservation priorities for marine turtles. *PLOS ONE* 6:e24510
- Whittier J, van de Merwe J, Limpus C, Ibrahim K (2003) Thermal biology of *Chelonia mydas* nests in eastern Peninsular Malaysia. In: Pilcher NJ (compiler) Proc 23rd Ann Symp Sea Turtle Biol Conserv. NOAA Tech Memo NMFS-SEFSC-536, p 129
- ▶ Wibbels T, Balazs GH, Owens DW, Amoss MS (1993) Sex ratios of immature green turtles inhabiting the Hawaiian Archipelago. *J Herpetol* 27:327–329
- ▶ Witt MJ, Hawkes LA, Godfrey MH, Godley BJ, Broderick AC (2010) Predicting the impacts of climate change on a globally distributed species: the case of the loggerhead turtle. *J Exp Biol* 213:901–911
- ▶ Wright LI, Stokes KL, Fuller WJ, Godley BJ and others (2012) Turtle mating patterns buffer against disruptive effects of climate change. *Proc R Soc B* 279:2122–2127
- Zug GR, Balazs GH, Wetherall JA, Parker DM, Murakawa SK (2002) Age and growth of Hawaiian green sea turtles (*Chelonia mydas*): an analysis based on skeletochronology. *Fish Bull* 100:117–127

Editorial responsibility: Paolo Casale,
Rome, Italy

Submitted: March 9, 2016; Accepted: August 17, 2016
Proofs received from author(s): October 10, 2016