Report

Current Biology

Environmental Warming and Feminization of One of the Largest Sea Turtle Populations in the World

Highlights

- We developed a novel method to estimate primary sex ratios in sea turtles
- We found extremely female-biased sex ratios in an important sea turtle population

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In Brief

Increasing incubation temperature impacts species with temperature-dependent sex determination such as green sea turtles. Jensen et al. combined genetic and endocrine techniques to show that an important green turtle population has produced primarily females for two decades, suggesting that complete feminization is possible in the near future.

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Current Biology
Report

Environmental Warming and Feminization of One of the Largest Sea Turtle Populations in the World

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SUMMARY

Climate change affects species and ecosystems around the globe [1]. The impacts of rising temperature are particularly pertinent in species with temperature-dependent sex determination (TSD), where the sex of an individual is determined by incubation temperature during embryonic development [2]. In sea turtles, the proportion of female hatchlings increases with the incubation temperature. With average global temperature predicted to increase 2.6°C by 2100 [3], many sea turtle populations are in danger of high egg mortality and female-only offspring production. Unfortunately, determining the sex ratios of hatchlings at nesting beaches carries both logistical and ethical complications. However, sex ratio data obtained at foraging grounds provides information on the amalgamation of immature and adult turtles hatched from different nesting beaches over many years. Here, for the first time, we use genetic markers and a mixed-stock analysis (MSA), combined with sex determination through laparoscopy and endocrinology, to link male and female green turtles foraging in the Great Barrier Reef (GBR) to the nesting beach from which they hatched. Our results show a moderate female sex bias (65%–69% female) in turtles originating from the cooler southern GBR nesting beaches, while turtles originating from warmer northern GBR nesting beaches were extremely female-biased (99.1% of juvenile, 99.8% of subadult, and 86.8% of adult-sized turtles). Combining our results with temperature data show that the northern GBR green turtle rookeries have been producing primarily females for more than two decades and that the complete feminization of this population is possible in the near future.

RESULTS AND DISCUSSION

Scientists are trying to describe, understand, and predict the effects of a warming planet on species and ecosystems, including shifts in species distribution [4, 5], changes in phenology [6], alterations in survival and fecundity rate [7, 8], reduced population size, and in some cases extinction [9]. For many species of reptiles such as crocodilians [10], some freshwater turtles [11], and all species of sea turtles [2], sex is determined by incubation temperature during embryonic development [12] (temperature-dependent sex determination, TSD). In sea turtles, cooler temperatures produce more male hatchlings while warmer temperatures produce more females [2, 12]; the incubation temperature that produces 50% of each sex [13] is defined as the pivotal temperature. The pivotal temperature is heritable and varies among species and populations [8], but the transitional range of temperatures that produce 100% males or 100% females spans only a few degrees Celsius [2]. Furthermore, extreme incubation temperatures not only produce female-only hatchlings but also cause high mortality of developing clutches [14]. With warming global temperatures and most sea turtle populations naturally producing offspring above the pivotal temperature [14], it is clear that climate change poses a serious threat to the persistence of these populations.

The basic sea turtle life history is similar in all species with only slight variation. In Australia, two genetically distinct breeding populations of green turtles are found at the opposite ends of the Great Barrier Reef: the southern Great Barrier Reef (sGBR) stock and the northern Great Barrier Reef (nGBR) stock, with virtually no nesting occurring along the middle part of the GBR [15] (Figure 1). Female and male green turtles along the east coast of Australia mate in the vicinity of their nesting beach, and female turtles can store sperm from a single mating for the entire nesting season [16]. Clutches of ~100 eggs are laid in nests dug on the beach, and females lay several clutches over the course of the nesting season. The eggs incubate for about 55 days [15]. After the hatchlings hatch and enter the sea, they spend several years in the open ocean before recruiting to coastal habitats along the Queensland coast, at which point

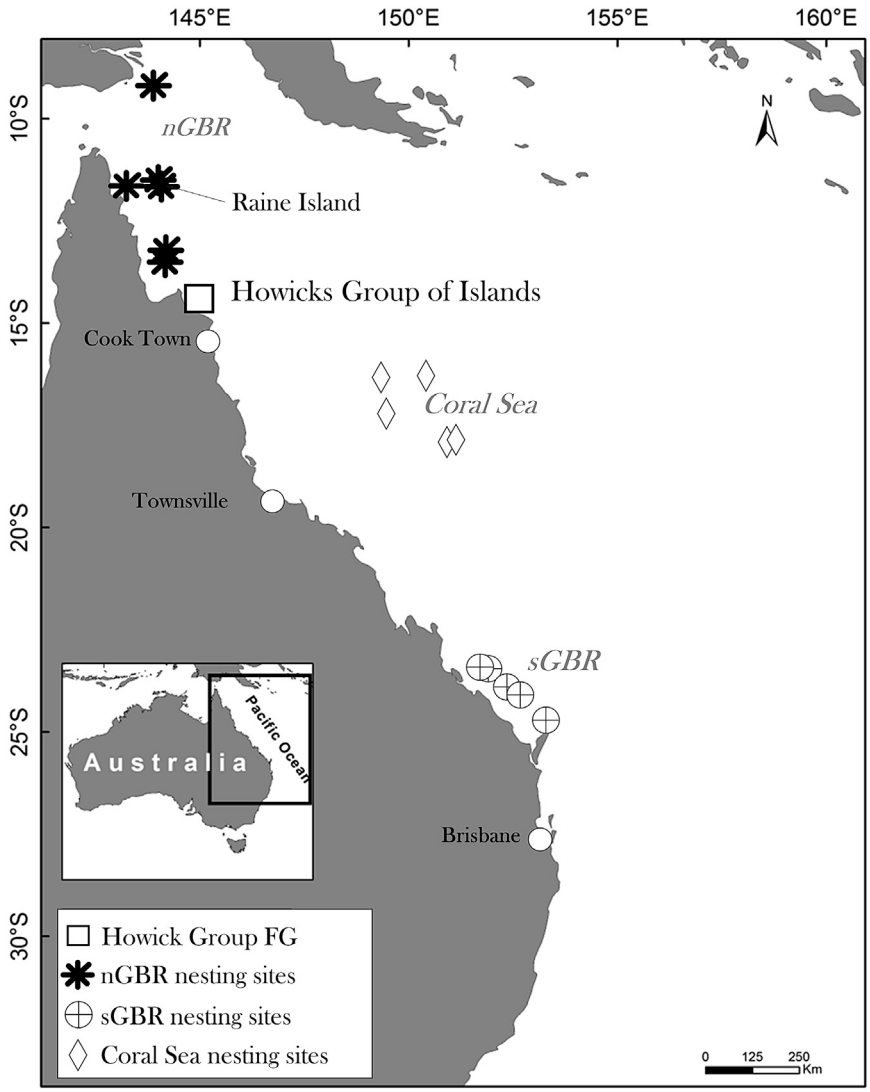

Figure 1. Green Sea Turtle Nesting and Foraging Sites within the Southwest Pacific
Howick Group foraging site (□) and regional rookeries (northern GBR [*], southern GBR [⊕], and Coral Sea [◇]) that are the main contributors to the Howick Group foraging aggregation.

they show strong fidelity to the same foraging area [17, 18]. At the onset of sexual maturity (~25+ years), female and male turtles migrate back to the vicinity of their natal beach to breed.

The nGBR is home to one of the largest green turtle populations in the world, with an estimated female population size in excess of 200,000 nesting females [15]. More than 90% of the females within the nGBR population nest at Raine Island and Moulter Cay (two small coral cays within the nGBR [15]). Despite the slightly increasing trend in the number of nesting females [19], early signs of population decline were identified in the mid-1990s when hatchling production on Raine Island was near zero [20]. Recent predictions of nGBR green turtle population trends suggest that low hatchling survival due to increasing water levels, altered weather patterns, and warming of habitat will further impact this turtle population in the future [21]. These effects are now detectable among foraging turtles along the GBR, where recruitment of juvenile green turtles from the nGBR is decreasing [18]. However, data are lacking at present for nest incubation temperatures and natural hatchling sex ratios produced in the nGBR stock, as well as information on the extent to which this population is already affected by increasing temperatures.

There are several challenges with directly estimating sex ratio at rookeries. At present, hatchling sex determination can be achieved reliably through histological examination of the gonads, but the sacrifice of live hatchlings carries ethical implications, and there is possible sampling bias in examining dead hatchlings found in nests [2, 22]. Furthermore, long-term incubation temperature data are lacking for nGBR rookeries [15]. Consequently, highly skewed hatchling sex ratios may have gone unnoticed for decades, with potentially negative effects. On the other hand, foraging aggregations represent cohorts of hatchlings from numerous rookeries throughout the region and reflect a range of life history stages. Therefore, characterizing sex ratios at foraging locations may offer greater insight into the overall population sex ratio [17]. Here, for the first time, we link individuals of known sex sampled within a foraging ground (FG) to their natal rookeries using genetic analysis and determine sex ratios produced at regional rookeries. These data represent many cohorts of turtles hatched at spatially distinct regional rookeries, providing a record of sex ratios produced at nesting beaches over the past 50+ years.

The Howick Group of islands is located in the nGBR (14°30′0″S; 144°58′10″E; Figure 1) and is used as a FG by a large number of green turtles. Both traditional tagging data and genetic analysis have shown that this foraging aggregation mainly comprises turtles from two genetic stocks, the nGBR and sGBR, which breed at opposite ends of the Great Barrier Reef [18] (Figure 1). Because sea turtles lack external sex-based traits until they reach sexual maturity, we used laparoscopic examination of the gonads and hormone analysis for sex identification of immature turtles, providing a unique opportunity to combine sex identification and genetic assignment data from the same population. Here we present an analysis of a large dataset of foraging green sea turtles (n = 411) to estimate (1) the sex ratios of different size classes (using curved carapace length [CCL] as a proxy for age) of green sea turtles at the Howick Group FG; (2) the natal origin of foraging turtles of different size classes and sex, using Bayesian mixed-stock analysis; and (3) the sex ratios of hatchlings produced at regional rookeries across recent history.

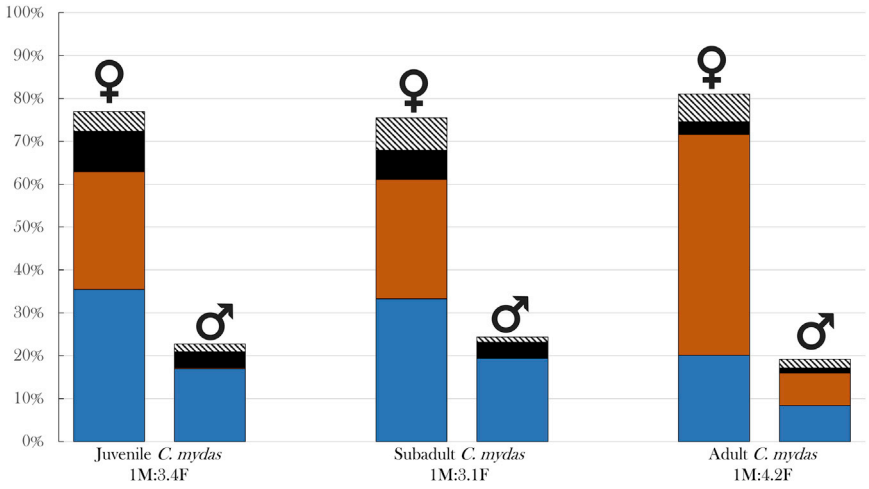

Figure 2. Nesting Beach Origin of Green Sea Turtles at a Northern Great Barrier Reef Foraging Ground

Relative proportions of male and female green sea turtles originating from the northern Great Barrier Reef (nGBR, red), and southern Great Barrier Reef/Coral Sea (sGBR/CS, blue) as determined via mixed-stock analysis. The remaining 22 rookeries are grouped into other rookeries (black), and haplotypes not identified at any rookery are classified as orphan haplotypes (hatched lines). See also Tables S1 and S2–S7.

Turtles were characterized as juvenile (35–65 cm CCL), subadult (65–85 cm CCL), or adult-sized (>85 cm CCL) (see STAR Methods). With natal origins combined, the sex ratios observed among turtles at the FG were female-biased for juvenile (1M:3.4F), subadult (1M:3.1F), and adult-sized turtles (1M:4.2F) (Figure 2). These ratios are similar to female-biased sex ratios determined at other FGs in the sGBR (Heron Island, 1M:2F [23]; Shoalwater Bay, 1M:1.74F to 1M:3.26F [24]) and nGBR (Clack Reef, 1M:2.1F to 1M:4.2F [25]). The mixed-stock analysis showed that female turtles across all size classes at the FG originated from both the nGBR (juveniles = 38%, subadults = 41%, adult-sized = 69%) and the sGBR/Coral Sea (juveniles = 49%, subadults = 49%, adult-sized = 27%) nesting populations as well as from rookeries outside the GBR (juveniles = 13%, subadults = 10%, adult-sized = 5% when combined), most of which originated from New Caledonia (Figure 1; Tables S2–S7). Finally, a small proportion of orphan haplotypes were found across all size/sex groups (5%–11%) (Figure 2; Table S1); orphan haplotypes have not yet been encountered within any nesting population and therefore are uninformative.

When the natal origins were taken into account, we found remarkable differences in sex ratios among size classes. Turtles originating from the nGBR showed extremely female-biased sex ratios, with 99.1% of juveniles, 99.8% of subadults, and 86.4% of adult-sized turtles being female (Table 1; Figure 2). Turtles originating from the sGBR, on the other hand, showed moderately skewed sex ratios, with 68% of juveniles, 65% of subadults, and 69% of adult-sized turtles being females. Our estimated sex ratios for the sGBR turtles are similar to reports from feeding grounds located in the sGBR (e.g., Moreton Bay: juveniles, 69% female; subadults, 54% female [26]) that are inhabited primarily by sGBR turtles [18] and increases confidence in our ability to estimate sex ratios produced in distant breeding populations.

Our results provide strong evidence for an extremely skewed female sex ratio in hatchlings produced at rookeries in the nGBR, while rookeries with cooler sand temperatures in the sGBR produce a less skewed sex ratio of approximately 1M:2.0F (Table 1). However, female bias in the nGBR is extreme only in immature (juvenile + subadult) turtles (>99% female) compared to adult-sized turtles (86.8% female), indicating that the proportion of females has increased in recent decades. To estimate the age span of different size classes, we assumed that juvenile turtles recruit into benthic foraging at around 30–40 cm CCL and 4–7 years of age. We used growth rates estimated for green turtles foraging at an adjacent feeding ground (Clack Reef) with a habitat similar to the Howick Group [27] to determine the age span for juveniles (4–16 years), subadults (13–23 years), and adult-sized turtles (>20 years).

We used recorded air and sea surface temperatures to estimate sand temperatures that nests would have experienced at the key rookeries in the nGBR from 1960 to 2016 following the model developed by Fuentes et al. [28] (Figure 3). Although we acknowledge that the temperature model has limitations in that it does not account for metabolic heating or nest depth, it clearly shows that green turtle nests at Raine Island and Moulter Cay were consistently incubated above the pivotal temperature (29.3°C [15]) since the early 1990s (Figure 3). Hence, the growth rates suggest that immature turtles at the Howick Group FG would have hatched within the past two decades during a time in which the nGBR experienced severe warming (Figure 3). Conversely, some adult turtles, born prior to the 1990s, would have experienced overall cooler temperatures during incubation (Figure 3), allowing for the production of more male hatchlings during this period.

Collectively, these results suggest that increased sand temperatures affect the sex ratios of the nGBR population such that virtually no male turtles are now being produced from these nesting beaches. Our findings add another dimension to the growing body of evidence that increasing temperatures are broadly affecting GBR ecosystems. Recent surveys revealed that 81% of the northern section of the GBR is severely bleached, whereas the cooler southern portion of the GBR is only mildly affected. The increased sea surface temperatures causing coral bleaching are correlated with increased beach sand temperatures, and thereby the incubation environment of sea turtle nests [28, 29].

Most sea turtle populations, including the ones nesting along the GBR, tend to nest during the hottest part of the year, suggesting that female bias is an adaptive trait maximizing the reproductive potential of the population [30, 31]. Species with TSD have existed for millions of years and coped with the selective pressures of a changing environment through adaptive changes of heritable traits [32]. In sea turtles, such adaptive changes

Table 1. Predicted Sex Ratios for Regional Rookeries Calculated for Three Size Classes of Green Sea Turtles

	Juveniles			Subadults			Adults		
Nesting Origin	p Female	95% CI	Sex Ratio	p Female	95% CI	Sex Ratio	p Female	95% CI	Sex Ratio
nGBR	0.99	(0.91–1.0)	1M:116F	0.99	(0.97–1.0)	1M:554F	0.87	(0.80–0.91)	1M:6.6F
sGBR/CS	0.67	(0.59–0.77)	1M:2.1F	0.65	(0.52–0.76)	1M:1.8F	0.69	(0.48–0.82)	1M:2.3F
Other	0.71	(0.36–0.94)	1M:2.4F	0.66	(0.22–0.34)	1M:1.9F	0.74	(0.10–0.98)	1M:2.9F
Orphan	0.77	NA	1M:3.4F	0.86	NA	1M:6.2F	0.75	NA	1M:3.1F

The probability (with 95% confidence interval [95% CI]) of being a female (p female) and estimated sex ratio by size class for each nesting population (rookery and orphan haplotypes) are provided. See also Tables S1 and S2–S7.

might include changes in pivotal temperature and thermal threshold [33], changes in the choice of nesting grounds [34], and/or adjusting the timing of nesting to earlier or later in the season [35, 36]. However, with slow growth to sexual maturity and strong natal homing, sea turtles are likely to be sensitive to rapid climate change. The strong genetic structure seen in mitochondrial (mt)DNA between green turtle nesting regions is a testament to strict natal homing in sea turtles and has resulted in deep demographic isolation of populations separated by only a few hundred kilometers (e.g., [37]). New colonization events are unlikely to be frequent enough to be significant in the course of a century or less. The process of natural selection takes place over the course of many generations [38], and for a long-lived species like sea turtles, such changes are likely to require centuries. With temperatures predicted to increase by several degrees in only a few turtle generations [3], many sea turtle populations like the nGBR will have little room to adapt to a rapidly changing climate.

Although increased breeding frequency, as well as polygynous behavior of male turtles, may help mitigate skewing offspring sex ratio [39], it is unknown how many (or what minimum proportion of) males is sufficient to sustain sea turtle populations. Furthermore, breeding male turtles have shown strong philopatry to courtship areas located in the vicinity of their natal beaches [40], suggesting that breeding males from other populations are unlikely to be present at nGBR courtship areas. While the nGBR and sGBR nesting females are highly differentiated for mtDNA, there is little differentiation at nuclear loci, suggesting that some level of male-mediated gene flow occurs between the two regions [41]. Most likely, inter-population mating occurs opportunistically when individuals from different populations mix at FGs or along migration corridors. Further studies are needed to quantify the degree of opportunistic inter-population mating, as well as gaining a better understanding of the operational sex ratio (OSR) at nGBR courtship areas. Recent studies have used drones to successfully distinguish between male and female turtles at mating areas [42] and shown that the increased breeding frequency of male turtles can result in stable OSRs, even for highly female-biased populations [42, 43].

While rising temperatures may initially result in increased female population sizes, the lack of male turtles will eventually impact the overall fertility of females in the population. For the nGBR, many of these questions will be answered as the adult population become increasingly female biased. As such, our study raises new concerns over the immediate threats of climate change to sea turtle populations. We need to learn more about how TSD species cope with rapid climate change, and we need to learn more about the male component in sea turtles and other TSD species to implement effective conservation efforts in the long term [44]. But more importantly, our study highlights the need for immediate management strategies aimed at lowering incubation temperatures at key rookeries to boost the ability of local turtle populations to adapt to the changing environment and avoid a population collapse—or even extinction [38].

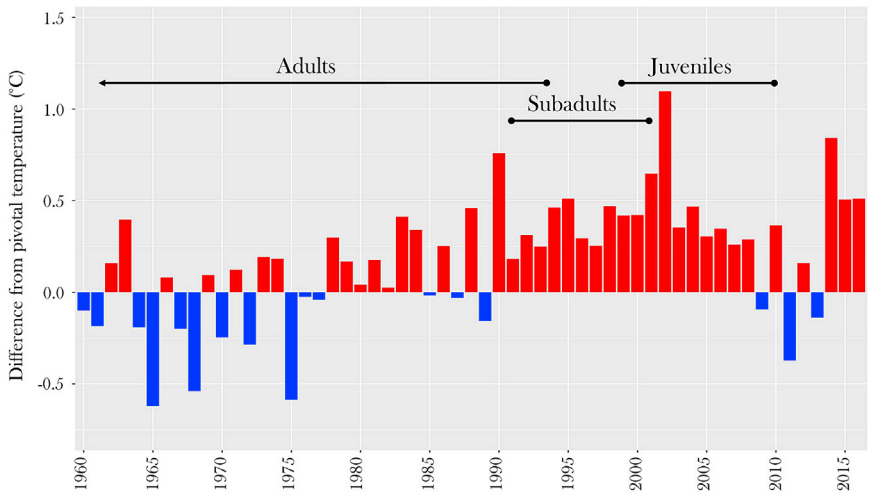

Figure 3. Estimated Sand Temperatures for Moulter Cay/Raine Island Rookeries

Estimated mean monthly sand temperatures for the middle of the nesting season (December–March) from 1960 to 2016. Bars show temperature above (red) and below (blue) the pivotal temperature of 29.3°C calculated for green turtles at Raine Island [15]. Also shown are the years in which adults, subadults, and juveniles were likely born at nGBR rookeries.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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- EXPERIMENTAL MODEL AND SUBJECT DETAILS
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  - Sample set
- METHOD DETAILS
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- QUANTIFICATION AND STATISTICAL ANALYSIS
  - Hormone assay
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- DATA AND SOFTWARE AVAILABILITY

SUPPLEMENTAL INFORMATION

Supplemental Information includes seven tables and can be found with this article online at https://doi.org/10.1016/j.cub.2017.11.057.

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AUTHOR CONTRIBUTIONS

Conceptualization, M.P.J., C.D.A., I.P.B., and T.E.; Methodology, M.P.J., C.D.A.., E.L.L., and T.E.; Investigation, M.P.J., C.D.A., T.E., I.P.B., W.A.H., and C.A.M.H.; Writing – Original Draft, M.P.J. and C.D.A.; Writing – Review & Editing, M.P.J., C.D.A., T.E., I.P.B., P.H.D., and C.A.M.H.; Funding Acquisition, M.P.J., C.D.A., I.P.B., P.H.D., and C.A.M.H.; Resources, I.P.B., C.A.M.H., and P.H.D.

DECLARATION OF INTERESTS

The authors have no interests to declare.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Biological Samples		
Green sea turtle samples (*Chelonia mydas*)	National Marine Mammal and Sea Turtle Research (MMASTR) Collection at the Southwest Fisheries Science Center, La Jolla, CA, USA	Lab ID #: see Table S1
Chemicals, Peptides, and Recombinant Proteins		
Exonuclease I and Shrimp Alkaline Phosphatase solution	Affymetrix/Thermo Fisher Scientific, CA, USA	Exonuclease I /CAT#70073X Shrimp Alkaline Phosphatase/CAT# 70092Z
ABI® Big Dye Terminator v3.1 Cycle Sequencing Kit	Applied Biosystems/Thermo Fisher Scientific, CA, USA	Cat# 4337456
Critical Commercial Assays		
Testosterone ELISA kit	ENZO Life Sciences, Plymouth, PA, USA	Cat# ADI-900- 065
Deposited Data		
Green turtle d-loop haplotypes	This study	GenBank: MG212505, MG212506, MG212507, MG212508, MG212509, MG212510, MG212511, MG212512, MG212513, MG212514, MG212515, MG212516, MG212517, MG212518
Oligonucleotides		
Primer: LCM15382 (5′ GCT TAA CCC TAA AGC ATT GG 3′)	Integrated DNA Technologies, San Diego, CA, USA	N/A
Primer: H950 g (5′ GTC TCG GAT TTA GGG GTT TG 3′)	Integrated DNA Technologies, San Diego, CA, USA	N/A
Software and Algorithms		
BAYES	[45]	https://www.afsc.noaa.gov/abl/MSA_software.htm
R	R Core Team	www.r-project.org
Magellan 3.11	Tecan Group Ltd., Männedorf, Switzerland	https://lifesciences.tecan.com/products/software/magellan_data_analysis_software
Geneious R8.1.9	[46]	https://www.geneious.com/

CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Dr. Michael Paul Jensen (michael.jensen@noaa.gov). There are no restrictions for use of the materials disclosed.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Animal use approval

All research in this study complied with all applicable animal welfare laws. Samples were collected under Queensland Turtle Research Project permit number G03/9866.1.

Sample set

A total of 337 wild foraging green turtles (*Chelonia mydas*) were sampled in July/August 2014 and 2015 from the Howick Group of islands (14°24'30.6"S 144°54'52.6"E) located in the northern part of the Great Barrier Reef Marine Park, Queensland, Australia. The Howick Group is made up by 16 reefs with large sandy reef platforms with patches of sea grass and algal meadows while the reef edges are dominated by coral growth.

Most turtles were hand captured from a small boat by rodeo-style methods in shallow (0.3–5 m) water. Turtles were randomly sampled by following transects across the reef platform. Different reefs and/or different sections of larger reefs were sampled

each day and were chosen based on weather and tide conditions. Once a turtle was spotted the boat would follow the turtles until caught and then return to the transect after sampling was completed. In addition, some juvenile turtles were hand captured in shallow water surrounding Ingram Island where the field camp was set. To ensure we were not sampling non-resident turtles undertaking their breeding migration all sampling was done in July/August well outside the breeding season (November-March).

We captured turtles of both sexes and all life stages (juvenile, subadult, and adult) in order to determine the genetic origin of turtles from each sex relative to their life stage. In addition, we used 74 previously published samples from adult green turtles sampled in July 2008 [21]. Turtles were divided into three size classes; juvenile (< 65 cm CCL), subadult (65-86 cm CCL), and adult-sized (> 86 cm CCL). The adult-sized turtles were based on the smallest size of nesting females at Raine Island [20]. Blood and skin samples were collected from each turtle for hormone and genetic analyses, respectively. The sex of a subsample of immature turtles (n = 139) were determined through laparoscopic examinations [47]. For adult-sized turtles, tail length was used to determine sex [48]. However, based on previous studies of green turtles we can assume that a small proportion of adult-sized turtles with short tails are likely immature females and ~2% are immature males [49]. All animals captured and sampled were healthy.

METHOD DETAILS

Hormone analysis and sex identification

We collected blood samples (maximum volume $\leq$ 3 mL/kg) from the dorsal cervical sinus [50] using a 1.5 inch, 21 gauge vacutainer needle (Becton, Dickinson and Company, Franklin Lakes, NJ) and a 6 or 10 mL sodium heparin vacutainer blood collection tube (Becton, Dickinson and Company) depending on the animal's mass. Samples were kept chilled on ice packs until centrifugation (1534 g for 10 min) after field work completion that day. Following centrifugation, plasma was aliquoted into 2 mL cryovials (Corning Inc., Corning, NY) and stored in a liquid nitrogen dry vapor shipper (Doble-34, MVE, Princeton Cryo, Pipersville, PA) while in the field and then at −80°C within the National Marine Mammal and Sea Turtle Research (MMASTR) Collection at the Southwest Fisheries Science Center, La Jolla, USA.

Samples were chosen at random using only the tissue as ID and, therefore, the researchers conducting the hormone analysis were blind to the sex of turtles identified via laparoscopy. Steroid hormones were extracted from plasma following the methodology described in Allen et al. [51]. In brief, steroid hormones were extracted from 50–500 μL of plasma using anhydrous ethyl ether (Fisher Scientific) extraction technique. Extracted samples were reconstituted in 250 μL of 0.01 M phosphate buffered saline (PBS, Sigma, St. Louis, MO) with 0.1% bovine serum albumin (BSA, Amresco, Solon, OH) prior to assay. A commercially available testosterone ELISA kit (Catalog # ADI-900- 065, ENZO Life Sciences, Plymouth, PA) that was previously validated for determining hormone concentration in green sea turtle plasma [51] was used to assess turtle sex. The assay validations included (1) demonstrating parallelism, where the slopes of plotted curves from serial dilutions of the hormone standard provided in the ELISA kit were compared to serial dilutions of pooled plasma extracts (n = 4) of unknown testosterone concentration, and (2) a matrix-interference test to examine if there was any potential interference caused by substances within our plasma samples, which are independent of specific antigen antibody binding. Standards (n = 7) of known testosterone concentration (1.9535 – 2,000 pg/mL) were prepared according to the assay kit protocol using PBS (with BSA) and, therefore, PBS (with BSA) was used as the 'zero' (B0) standard. The sensitivity of the assay was 2.0 pg/mL and the effective sensitivity of the assay is 1.2 pg/mL after correction for plasma volume, acetone volume, extraction efficiency, reconstitution volume, and dilution.

Testosterone concentrations were determined in each extracted plasma sample and all samples were quantified in duplicate (Table S1). All samples were assayed undiluted following reconstitution in PBS (with BSA). For samples that had testosterone concentrations above the detection limit of the assay, we diluted (in PBS with BSA) a subsample of the plasma sample extract and obtained a reliable testosterone concentration on a subsequent assay. A sample would be re-assayed if the variation between duplicate analysis of the same sample was >10% (n = 14). We also re-extracted and re-assayed samples where the testosterone concentrations were anomalous (n = 16; e.g., a sample had unusually high or low T concentrations for that size/sex of turtle). Mean extraction efficiency was 90.7% and the mean intra- and interassay coefficients of variation were 6.7% and 17.4%, respectively (n = 11 assays).

A Tecan spectrophotometer (Model: Sunrise, Phenix Research Products, Candler, NC, USA) was used to read the optical density within each well of the ELISA plate. The resulting testosterone concentrations (pg/mL) were computed using a five-parameter logistic curve fitting program (Magellan 3.11, Tecan Group Ltd., Männedorf, Switzerland). To assess the accuracy of determining the sex using hormone assay the sex of 139 juvenile and subadult turtles were determined using laparoscopic examination.

Genetic analysis

DNA was extracted from tissue samples using a modified sodium chloride precipitation protocol [52]. Tissue was digested by Proteinase K (10 mg/ml) at 37°C overnight in TEQ buffer (pH 9.0; Tris base, EDTA, NaCl) and of 10% sodium dodecyl sulfate (SDS). Proteins were removed by a precipitation process using NaCl (6M), followed by a 100% ethanol precipitation of the DNA at −20°C overnight. DNA pellets were dried and eluted in TE buffer (Tris, EDTA).

We amplified ~800 bp of the mtDNA control region (d-loop) using primers LCM15382 (5′ GCT TAA CCC TAA AGC ATT GG 3′) and H950g (5′ GTC TCG GAT TTA GGG GTT TG 3′) [53] and standard PCR reagents (MilliQ water, 10mM dNTPs, Taq DNA polymerase and 10x ThermoPol Reaction Buffer). Samples were amplified in a 25 μl PCR reaction and were carried out on MJ Research or Bio Rad PTC-100s or Applied Biosystems® 2720 thermocyclers. The PCR parameters were an initial 2 min DNA denaturation at 90°C, followed by 30 cycles of 50 s denaturation at 94°C, 50 s annealing at 56°C, and 1 min extension at 72°C, with a final 5 min extension at

72°C. The PCR products were confirmed visually by electrophoresis on 2% agarose gels stained with ethidium bromide. Purification of PCR products was done by combining 5 μl of PCR product with 2 μl of an Exonuclease I and Shrimp Alkaline Phosphatase solution (Affymetrix, CA, USA). Both strands were sequenced using recommended protocols for a Big Dye® Terminator v3.1 Cycle Sequencing Kit on the Applied Biosystems Inc. (ABI/Thermo Fisher, CA, USA) model 3730 automated genetic analyzer. To detect possible contamination and/or sequencing errors negative controls were used for both the DNA extraction and the PCR reactions (12% of samples). Positive controls were included by rerunning 10% of all samples (randomly selected) for both the PCR and the sequencing reaction. In addition, new haplotypes were re-extracted and re-sequenced to confirm that they were indeed new haplotypes. No evidence of contamination or sequencing error was detected in this study.

Final sequences were analyzed and edited using the program Geneious created by Biomatters (https://www.geneious.com/; [46]). Sequences were aligned using Clustal X [54] as implemented in Geneious version 6.0.2. To identify individual haplotypes we performed searched sequences against the GenBank database as well as the SWFSC sea turtle haplotype database. For comparison to published haplotypes we cropped all sequences to ~766 bp.

Temperature data

Sea surface (SST) and air (AT) temperature data were monthly averaged over 1° x 1° spatial scale (SST: Met Office Hadley Centre, http://hadobs.metoffice.com AT: International Comprehensive Ocean-Atmosphere Data Set; ICOADS). SST and AT were downloaded from a NOAA data server (http://coastwatch.pfeg.noaa.gov/erddap/index.html) for the nGBR area defined by 7.5S, 12.5S, 142.5E, and 144.5E. Regression analyses between temperature (SST or AT) and time (from December through March of each year) was conducted using linear, broken-line, and non-linear models using the segmented [55] and mgcv [56] packages in R (v. 3.4.1; R Core Team). For the non-linear models, we used the generalized additive model with splines and with dimensions of the basis (k parameter in gam function within the mgcv package) between 3 and 6. We used AIC (Akaike Information Criteria; [57]) to compare models and selected the most parsimonious model (minimum AIC value) for SST and AT independently. GAMs with dimensions of the basis 6 and 4 were considered best for SST and AT, respectively. These models, then, were used in the following computations.

Monthly sand temperatures at Moulter Cay, were computed using the predicted values from the best models for SST and AT and regression equations provided in Fuentes et al. [58], which were

$$\cdot \text{Moulter Cay} = -0.236 * \text{SST} + 1.022 * \text{AT} + 6.915$$

Given that no data were available for Raine Island, we chose Moulter Cay as a proxy for the nGBR. Moulter Cay is located within 20 km of Raine Island, has a similar beach profile and together they host the largest portion of nesting green turtles in the nGBR with approximately 90% of the nesting occurring at these two islands.

Estimated mean monthly sand temperatures for the middle of the nesting season (December-March) from 1960 to 2016 were plotted against a pivotal temperature of 29.3°C calculated for green sea turtles at Raine Island [15] (Figure 3).

QUANTIFICATION AND STATISTICAL ANALYSIS

Hormone assay

A cross-validation analysis was conducted by predicting the sex of each individual using a model developed with all data except the individual. The accuracy of the predictive power of the model was determined by how many of the 139 individuals with known sex (from laparoscopic examination) were assigned to a correct sex. The accuracy of the model was 97.1%, where 135 individuals were assigned correct sex given their testosterone values. In addition, sex was determined for 26 immature turtles using only laparoscopy. Hence, only 74 immature turtles out of 232 were sexed using the ELISA alone, resulting in an overall error rate for determining the sex of immature turtles of ~1% (Table S1).

Mixed-stock analysis

A Bayesian approach using the program BAYES [45] was used to estimate the contribution of the baseline rookeries to the foraging turtles. This method has previously been shown to produce accurate estimates of stock origin for green turtles along the GBR (see [18]). The rookery reference data included 25 published regional rookeries [18]. Six individual mixed-stock analyses were run for each sex and size specific group (i.e., juvenile males). For each group the analysis was run using four chains each with a different starting point. Each chain was run for 50,000 iterations with the first 25,000 runs discarded as burn-in. The gelman-Rubin shrink factor was calculated to ensure that all chains had converged [59]. The model was run using populations size priors thereby assuming that larger rookeries contribute more turtles than smaller rookeries. Results were grouped into three regional nesting origins as follows; region 1 = nGBR, region 2 = sGBR and Coral Sea combined, and region 3 = all other rookeries combined (see Tables S2–S7). The grouping of sGBR and Coral Sea was based on genetic similarity that could otherwise lead incorrect estimates for the two stocks individually.

Sex ratio estimation

The sex ratios of a specific stage class (i.e., juveniles) for each of the three rookery origins was calculated using expected numbers of males and females for each age class and a rookery. Given an estimated contribution from the k-th rookery, the expected number

of individuals of each sex for the k-th rookery was computed as $n_{i,j,k} = p_{i,j,k} \times n_{i,j}$, where i = male or female, and $n_{i,j}$ is the total number of observed (or assumed) individuals of the i-th sex and the j-th stage. The proportion of each sex, then, was computed ($p_{i,j,k} = n_{i,j,k}/(n_{f,j,k} + n_{m,j,k})$). Confidence intervals for the estimates were computed using 95% confidence intervals for the estimated contributions of rookeries (Tables S2–S7). For example, the estimated contribution of the nGBR to juvenile females was 0.35 and it was 0.01 for juvenile males. There were 131 juvenile females caught. Therefore, the expected numbers of female and male juveniles were 46 and 0.4, respectively. The proportion of females, then was 46/46.4 = 0.99.

DATA AND SOFTWARE AVAILABILITY

The raw dataset for this study is provided as Table S1.

The accession numbers for the newly discovered mtDNA haplotypes reported in this paper are GenBank: MG212505, MG212506, MG212507, MG212508, MG212509, MG212510, MG212511, MG212512, MG212513, MG212514, MG212515, MG212516, MG212517, MG212518 (https://www.ncbi.nlm.nih.gov/genbank/).