

SEX RATIO PREDICTION OF JUVENILE HAWKSBILL SEA TURTLES (*ERETMOCHELYS IMBRICATA*) FROM SOUTH FLORIDA, USA

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Abstract.—All sea turtles exhibit temperature-dependent sex determination, a process in which the incubation temperature of the nest determines the sex of the offspring. Given the potential effects of climate change worldwide, monitoring sea turtle population sex ratios has become a priority, especially for these threatened or endangered species. In this study, we examined the sex ratio of an aggregation of juvenile Hawksbill Turtles (*Eretmochelys imbricata*), a critically endangered species, at a coral reef in southern Florida, USA. This is the most northerly juvenile Hawksbill aggregation in the Atlantic Ocean for which this type of study has been completed, and the first one within the continental USA. We hand-captured turtles and collected blood samples from 69 individuals over a period of two years for sex determination using a testosterone radioimmunoassay (RIA). The RIA was calibrated with serum samples from juvenile Hawksbills whose sex was confirmed by laparoscopy, from Mona Island, Puerto Rico and Caribbean Panama. Our results indicate a significant female bias in this population (2.37:1), which has also been observed in several previous studies of juvenile Hawksbill turtle sex ratios.

Key Words.—Cheloniidae; *Eretmochelys imbricata*; foraging; Hawksbill Turtles; Reptilia; RIA; sex ratio; USA

INTRODUCTION

Hawksbill Turtles, *Eretmochelys imbricata* (Fig. 1), are found in tropical and sub-tropical seas worldwide, and are classified as critically endangered under the IUCN Red List of Threatened Animals (Baillie and Groombridge 1996). As with all marine turtles, the gender of offspring is determined by the temperature experienced during embryonic development, a process called temperature-dependent sex determination (Bull and Vogt 1979; Vogt and Bull 1984). In the case of all sea turtles studied, temperatures above the pivotal temperature produce mostly females, and slightly colder temperatures produce mostly males (Yntema and Mrosovsky 1980; Mrosovsky 1994; Wibbels et al. 2000). Temperature-dependent sex determination can have both evolutionary and conservation implications by creating biased sex ratios within populations (Mrosovsky and Yntema 1980; Morreale et al. 1982; Mrosovsky 1994). Numerous studies have found a diversity of sex ratios of hatchling and juvenile sea turtles including some that were highly biased, more often towards females than males (e.g., Mrosovsky and Provancha 1989; Marcovaldi et al. 1997; Hanson et al. 1998; Wibbels et al. 1999; Casale et al. 2000; Geis et al. 2005). Such skewed sex ratios are of interest because they do not conform to the 1:1 ratio predicted by

evolutionary theory (Fisher 1930). Furthermore, sex ratios should be monitored for the potential effects of climate change on sea turtle populations worldwide, so that the impact of changing sex ratios can be considered in future conservation strategies (Glen and Mrosovsky 2004; Hawkes et al. 2007).

Our goal was to examine the sex ratio of an aggregation of juvenile Hawksbill Turtles that inhabit the coral reefs of northern and central Palm Beach County, Florida, USA. This is the most northerly



FIGURE 1. A sub-adult Hawksbill Turtle captured along the coral reefs offshore of Palm Beach County, Florida, USA (Photographed by Mark Conlin).

Hawksbill Turtle population in the Atlantic Ocean for which this type of study has been completed. Though known to recreational SCUBA divers for decades, little documentation exists concerning the population structure of Hawksbills in south Florida. In contrast to more southerly locations in the Caribbean, Florida does not have an established rookery, though occasional nesting has been reported from the Florida Keys northward through Volusia County (Meylan and Redlow 2006). However, juvenile and sub-adult individuals are regularly encountered in nearshore reef habitats of several counties on the east coast (Meylan and Redlow 2006; Lawrence Wood, pers. obs.). Occasionally, adult Hawksbill Turtles are also found in the study area, but they were not included in the present study because adult sea turtles undergo reproductive migrations that can lead to sex-bias depending on the capture location and time of year. Moreover, testosterone levels can be elevated in migratory adult females, resulting in overlaps of testosterone ranges between males and females, which can be difficult to interpret, and could therefore lead to sex determination errors (Wibbels et al. 1991).

MATERIALS AND METHODS

Capture and sampling procedure.—We collected samples in spring and summer 2005, and from spring 2006 to spring 2007. We hand captured Hawksbill Turtles during daytime SCUBA dives along the coral reefs offshore of northern and central Palm Beach County, Florida, USA in waters ranging from 1.8 to 27.4 m in depth. We recorded surface water temperature for each capture using a dive computer. Once captured, we placed the turtles on a research vessel for sampling and tagging. We used standard straight carapace length (SCLnt) measurements to estimate the maturity status of individuals, and we only analyzed turtles with SCLnt < 70 cm for sex determination (for size at maturity, see Meylan and Redlow 2006 for discussion). Before release, we extracted blood from the cervical sinus (Owens and Ruiz 1980) using 3.81 cm, 20 gauge needles and 7 mL Monoject® lithium heparin vacutainer blood collection tubes (Tyco Healthcare Group, Mansfield, Massachusetts, USA), and samples were immediately placed on ice. We centrifuged the blood for at least 10 minutes no later than four hours after collection. We then pipetted the plasma into 5 mL cryovials, and we stored samples in a standard industrial freezer. Samples were later shipped on dry ice to the Grice Marine Laboratory, where they were stored at -80°C until analysis.

Testosterone assay.—Because of the absence of secondary sexual characteristics in juvenile sea turtles, measurement of plasma testosterone concentrations has been developed and widely used to evaluate the sex of

juveniles, and subsequently the sex-ratio of specific sea turtle populations worldwide (Owens et al. 1978; Wibbels et al. 2000). In the present study, we analyzed testosterone levels by RadioImmunoAssay (RIA) as previously described by Wibbels et al. (1987). For each sample, 500 µL of plasma was extracted with 4 mL of anhydrous diethyl ether, dried under nitrogen gas, and resuspended with 1 mL of acetone. Two 400-µL aliquots were pipetted from the 1 mL of acetone, and the tubes were dried in air overnight. The following day, we reconstituted each tube with 100 µL of tris/gel buffer, and incubated them in a water bath for 30 minutes at 37°C. At this point, tubes containing 100 µL of testosterone standard solution with concentrations ranging from 19.5 to 1250 pg/mL were prepared in duplicate. Following incubation, we added 200 µL of testosterone antibody (# T3-125, lot # 338A, purchased from Endocrine Sciences, Calabasas Hills, California, USA) to all tubes (standards and samples), as well as 100 µL of tritiated testosterone (~6500 cpm; PerkinElmer Life and Analytical Sciences, Inc, Boston, Massachusetts, USA). The tubes were incubated overnight at 4°C. After incubation, we added 1 mL of dextran-coated charcoal to all tubes except those used to determine total counts. All tubes were incubated for 15 minutes at 4°C, and centrifuged at 2,300 rpm at 4°C for 15 minutes. The supernatant was poured into scintillation vials and 4 mL of Ecolume scintillation cocktail were added to each vial. The vials were counted for 60 seconds with a Wallac 1409 liquid scintillation counter. We calculated the testosterone concentrations in pg/mL from the counts using the standard curve. Values were corrected by multiplying the volume extracted by the extraction efficiency and the fraction aliquoted from the reconstituted sample (40%). We calculated the extraction efficiency by adding approximately 6500 cpm (in a 100 µL volume) of tritiated testosterone to six-500 µL and six-250 µL serum sub samples prior to extraction. Extraction efficiencies averaged 91.8% for the 500 µL-samples, and 94.6% for the 250 µL-samples. We extracted a Loggerhead (*Caretta caretta*) control sample four times in each assay to evaluate intraassay and interassay variability.

We calibrated the testosterone RIA used for south Florida Hawksbills with 17 plasma samples from juvenile Hawksbill Turtles whose sex was determined by laparoscopic examination of the gonads following the method of Owens (1999). We performed laparoscopy on 17 juvenile Hawksbills with accompanying blood samples from Mona Island (n = 14), Puerto Rico (Diez and Van Dam 2003), and Bocas Del Toro Province (n = 3), Panama (Geis et al. 2003). We analyzed these samples using the same RIA assay as described above, which provided specific testosterone concentration ranges for known males and females that were then used

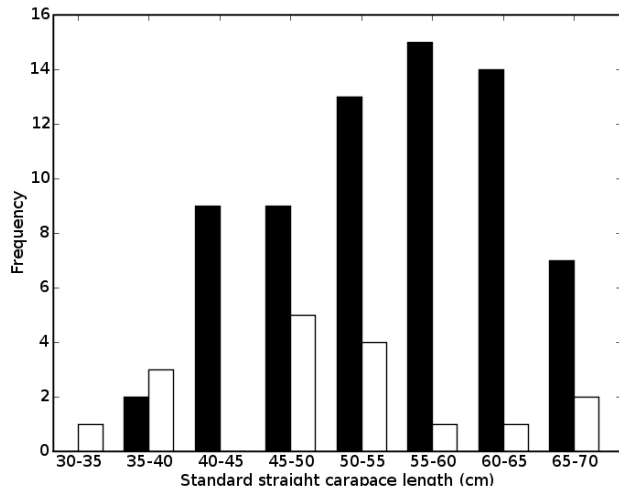


FIGURE 2. Frequency distribution of standard straight carapace lengths (notch to tip in cm) of juvenile Hawksbill Turtles from the study site in Palm Beach County, Florida, USA (black bars, n = 69) and from the Caribbean region (white bars, n = 17). Hawksbills from the Caribbean region were used for the calibration of the RIA.

to predict the sex of juvenile Hawksbill Turtles from south Florida.

Statistical analysis.—We performed statistical tests using the software R version 2.2.1 (R Development Core Team, 2005. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0, URL <http://www.R-project.org>). Assumptions for normality and equality of variances were tested using Shapiro-Wilk’s test and Bartlett’s test, respectively. We compared the straight carapace lengths of south Florida Hawksbills and the Caribbean Hawksbills used for calibration using a *t*-test. We also compared the resulting sex ratio for this juvenile south Florida Hawksbill population to a 1:1 ratio using the Goodness of fit (G) test. All testosterone concentrations are reported in pg/mL ± 1 standard error, and $\alpha = 0.05$ for all tests.

RESULTS

Size composition.—Straight line carapace lengths of the 17 turtles used for the calibration of the testosterone assay ranged from 34.0 to 66.2 cm (mean ± SD = 49.7 ± 9.6 cm). We sampled 69 individual juvenile Hawksbills from south Florida, and their straight carapace lengths ranged

from 35.7 to 68.4 cm (mean ± SD = 54.7 ± 8.25 cm). Although their ranges overlapped greatly, the straight carapace lengths of south Florida Hawksbills were significantly larger than those of Hawksbills used for the calibration experiment ($t = -2.10$, $df = 85$, $P = 0.039$) (Fig. 2).

Calibration of the testosterone assay.—The intra-assay coefficient of variation was 11.3%. Of the 17 samples from turtles whose sex was verified by laparoscopy, six were from juvenile females, and 11 were from juvenile males. Testosterone levels of females and males did not overlap. They ranged from 134.4 to 260.8 pg/mL for females (mean = 168.0 ± 19.8 pg/mL), and from 720.5 to 13,181 pg/mL (mean = 3,315.4 ± 1,132.6 pg/mL) for males.

Sex ratio of juvenile Hawksbills from South Florida.—Three assays were necessary to analyze all 69 samples from south Florida. The intra-assay and inter-assay coefficient of variations were 12.5% and 13.8%, respectively. Overall testosterone concentrations ranged from 67.3 to 3,815 pg/mL. Based on the testosterone ranges of females and males from the calibration assay, we predicted that 45 samples came from females, and 19 from males. Five samples were not assigned a sex because their testosterone concentration fell in the intermediate range, below the lower limit of the male’s range, and above the highest limit of the female’s range (from 260.8 to 720.5 pg/mL) (Fig. 3). The average testosterone concentration for

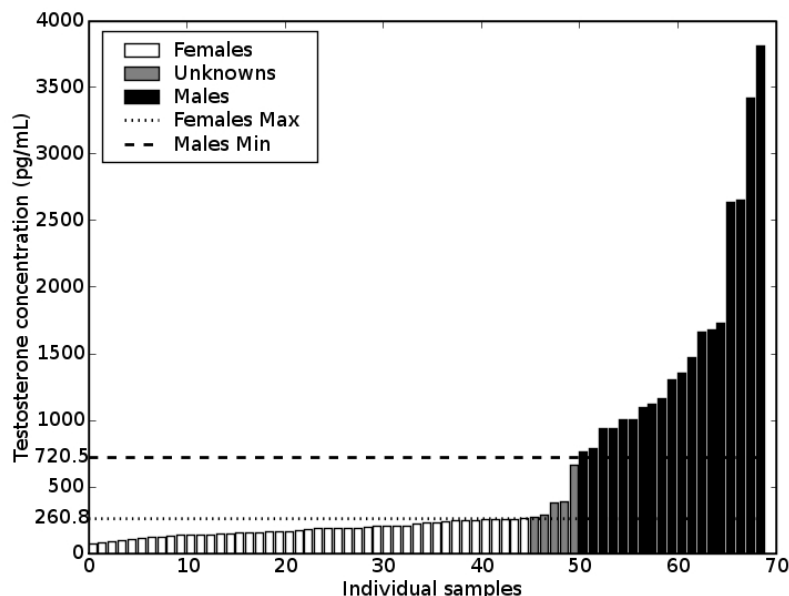


FIGURE 3. Testosterone concentrations (pg/mL) of plasma samples from juvenile Hawksbill Turtles captured in Palm Beach County, Florida, USA. Sex was determined based on the calibration assay. White bars represent female levels, black bars represent male levels, and gray bars represent samples for which sex was undetermined. Two lines show threshold testosterone levels for juvenile female and male Hawksbills based on the calibration assay.

TABLE 1. Summary of previously published sex ratios of juvenile Hawksbill Turtles worldwide, including our study. Sex ratios significantly different from a 1:1 ratio ($P < 0.05$) are marked with an asterisk.

Location	Time period	Sample size	Sex Ratio (F:M)	Method of Sexing	Reference
Southern Great Barrier Reef, Australia	May 1969 - April 1988	109	2.57:1*	Laparoscopy/necropsy	Limpus, 1992
Dominican Republic	April 1996 - April 1998	143	2.71:1*	Testosterone RIA	Leon and Diez, 1999
Mona Island, Puerto Rico	1993 - 1995	120	0.8:1	Laparoscopy/Testosterone RIA	Diez and Van Dam, 2003
Buck Island Reef, US Virgin Islands	April 1995 - October 1999	72	4:1*	Testosterone RIA	Geis et al., 2003
South Florida, USA	2005 - 2007	69	2.37:1*	Testosterone RIA	This paper

juvenile females from south Florida was 177.8 ± 7.8 pg/mL (range: 67.3-260.5), and $1,607.7 \pm 204.1$ pg/mL (range: 766.3-3,815) for juvenile males. Among the five unassigned samples, two were below 300 pg/mL, two were between 300 and 400 pg/mL, and one was between 600 and 700 pg/mL. The resulting female to male sex ratio was 2.37:1 (excluding samples that were not assigned a sex), which was statistically different from a 1:1 ratio ($G = 10.9$, $P < 0.001$).

DISCUSSION

The sex ratio found in this study was significantly skewed towards females (2.37:1). The sex ratio of this Hawksbill population may be slightly more female-skewed if most of the samples that fell in the intermediate “unknown” testosterone range were females. Indeed, two of those samples were < 300 pg/mL, which is only a few pg/mL larger than the observed female range. Two were between 300 and 400 pg/mL, which is still far below the observed male range, so we suspect these unassigned individuals may have also been females. Only one sample classified as unknown was just slightly below the male testosterone range. If four out of the five unassigned samples were females, the sex ratio would be 2.45:1 (female to male). A larger sample size for the calibration assay could have allowed the extension of the male and female ranges, and the inclusion of those five samples in one of the two sex categories. In any case, the sex ratio of this population would remain significantly female-biased.

The average straight carapace length of Hawksbill Turtles caught in south Florida was significantly larger than that for the turtles used for the calibration assay, which could explain why females from south Florida had slightly higher testosterone concentrations than females in the calibration group. Although the sampling method could have introduced a bias by capturing one sex more than the other due to sex-specific behaviors, no study has ever shown that juvenile Hawksbills exhibit a sex-related bias in diet, habitat use, or diving/surfacing behavior.

Therefore, there is no reason to expect that the sampling technique used in Florida (hand capture during SCUBA dives) favored the capture of one sex over the other, leading to this female-bias. Also, the average surface water temperature during sampling was 27.1°C (range: 23.3-29.4), which is above the minimum water temperature for the RIA reliability, as recently documented for juvenile Loggerheads (Braun-McNeill et al. 2007). Thus, the significant female-biased sex ratio predicted in this study is expected to represent accurately this juvenile Hawksbill aggregation.

Several other studies have also reported a female bias in their juvenile Hawksbill feeding aggregations in southwestern Dominican Republic (Leon and Diez 1999), around Buck Island in the U.S. Virgin Islands (Geis et al. 2003), and even in Australia, around Heron Island, at the Southern end of the Great Barrier Reef (Limpus 1992). In one case only, a non-biased sex ratio was predicted at Mona Island, Puerto Rico (Diez and Van Dam 2003) (see Table 1). It is not known, nor well understood, why such skewed sex ratios exist, and if there is an ecological and/or evolutionary advantage to such bias. Fisher’s theory (1930) predicts, given equal costs of producing either gender, that the sex ratio should be 1:1 at the time parental investment ends. In the case of sea turtles, the sex of the offspring is determined by the incubation temperature on the beach, well after females have laid their eggs and returned to the sea, and the parents have no direct control over the sex ratio. Such conditions make it difficult to understand the sex ratio dynamics of sea turtles, especially because the samples used in sex ratio studies typically represent a small sample in time and space from one population (hatchling studies) or a small sample in time from multiple nesting beach populations (foraging aggregations). Nevertheless, gathering information on sex ratios of juvenile sea turtle aggregations worldwide is necessary to understand better their temporal and spatial evolution, and to improve conservation programs. Indeed, if global warming causes additional bias in sea turtle sex ratios, males

might become the limiting resource. In a worst-case scenario, global warming could lead to the elimination of male offspring production altogether, as predicted by Janzen (1994) for another turtle species. Clearly, highly skewed sex ratios could have long-term impacts on the reproductive ecology and the evolution of sea turtles, including effects on multiple paternity, sperm competition, and fertility (Lovich 1996). At the population level, highly skewed sex ratios could potentially lead to an Allee effect if the population size of the reproductively active individuals was too small (Stephens et al. 1999). To test this hypothesis, more work would need to be done to assess the size and sex ratio of breeding populations, which is difficult to implement due to the wide dispersal of the adults, and to variable remigration intervals. Assessment of the population would have to be done over a long time period to reduce these variabilities.

In conclusion, this study is the first one focusing on juvenile Hawksbill Turtles in the continental USA, and therefore provides the first sex ratio of a juvenile Hawksbill aggregation in this area. Preliminary results for mtDNA analyses revealed at least six distinct Caribbean haplotypes (Bass et al. 1996), indicating that individuals from this population originate from different Caribbean nesting colonies as has previously been reported by Bass (1999) and Bowen et al. (2007) for foraging aggregations of juvenile Hawksbill Turtles elsewhere in the West Atlantic. Thus, the sex ratio of this south Florida Hawksbill population is a product of the primary sex ratio produced at multiple rookeries in the Caribbean. Further genetic study will suggest which nesting populations are represented.

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LAWRENCE WOOD has been active in sea turtle biology and conservation in South Florida for over 19 years. During this time, he has coordinated sea turtle nesting surveys, developed a sea turtle rehabilitation facility, and has created numerous educational programs related to marine science and conservation. His lifelong interest in herpetology took him to Miami University of Ohio for several years before completing his undergraduate degree in Ecology at Florida Atlantic University (FAU). He then earned his Master's degree in Environmental Science, also at FAU. Mr. Wood is currently involved in the development of several new marine research projects related to Florida's hawksbill turtles and their habitats.



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