Conservation Physiology

Volume 9 • 2021 10.1093/conphys/coab072

SEB SOCIETY FOR EXPERIMENTAL BIOLOGY

Research article

Biomarkers of reproduction in endangered green sea turtles (*Chelonia mydas*) nesting at Tortuguero, Costa Rica

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Understanding the timing of vitellogenesis is essential for identifying threats to the reproductive success of endangered oviparous vertebrate species, such as sea turtles. We measured concentrations of testosterone (T) and vitellogenin (VTG) in green sea turtles (*Chelonia mydas*) nesting at Tortuguero, Costa Rica, as biomarkers of ovarian development. Testosterone concentration increased from the first to second month and VTG concentration increased at the third week of sampling. These results show that Tortuguero green sea turtles were still producing both biomarkers early into the nesting season. VTG concentration was negatively correlated with female weight, suggesting that larger females start nesting earlier at Tortuguero and that we may have sampled larger females further into their reproductive cycle.

Key words: Conservationendocrinologyreproductive biologysea turtlesTortuguero

Editor: Steven Cooke

Received 3 October 2020; Revised 22 March 2021; Editorial Decision 6 August 2021; Accepted 9 August 2021

Cite as: Bruno RS, Machado JAR, Guzman GRB, Loria JIR, Valverde RA (2021) Biomarkers of reproduction in endangered green sea turtles (*Chelonia mydas*) nesting at Tortuguero, Costa Rica. *Conserv Physiol* 9(1): coab072; doi:10.1093/conphys/coab072.

Introduction

Vitellogenesis is a similar process in most oviparous reptiles, during which lipids and proteins are mobilized from the body's stores and progressively added to developing ovarian follicles, forming the egg yolk that nurtures growing embryos (Ho *et al.* 1980, 1982; Hamann *et al.* 2003; Polzonetti-Magni *et al.* 2004). The series of physiological processes occurring during vitellogenesis are regulated by specific gonadal and non-gonadal hormones (Urist and Schjeide 1961; Callard and Klotz 1973; Licht *et al.* 1980; Ho *et al.* 1980, 1982; Ho 1987; Wibbels *et al.* 1990; Heck *et al.* 1997; Mosconi *et al.* 1998; Hamann *et al.* 2003; Polzonetti-Magni *et al.* 2004).

Oestrogens induce growth and structural modifications in the liver of snakes and lizards that are associated with the production and secretion of proteins (reviewed by Ho 1987). Evidence indicates that estradiol 17β (E_2) induces the production of vitellogenin (VTG), which is the main protein sequestered into growing ovarian follicles during vitellogenesis and cleaved into the main proteinaceous components of egg yolk (Bergink and Wallace 1974; Ho *et al.* 1981; Ho 1987; Hamann *et al.* 2003; Polzonetti-Magni *et al.* 2004).

Because yolk deposition into ovarian follicles is crucial to reproductive success of oviparous vertebrates (Polzonetti-Magni *et al.* 2004), the importance of studying endocrine

mechanisms that control vitellogenesis has been highlighted by several studies, many of which focused on threatened and endangered sea turtle species (Licht *et al.* 1980; Owens and Morris 1985; Wibbels *et al.* 1990; Rostal *et al.* 1998; Hamann *et al.* 2003; Polzonetti-Magni *et al.* 2004; Sifuentes-Romero *et al.* 2006; Smelker *et al.* 2014; Myre *et al.* 2016). However, there are significant gaps regarding the hormonal regulation of the onset and completion of vitellogenesis in reptiles (Hamann *et al.* 2003; Polzonetti-Magni *et al.* 2004). Research is lacking on the variation of such hormones, associated proteins and requisite body condition during the reproductive cycle of free-ranging migratory reptiles, such as sea turtles (Hamman *et al.* 2003).

Sea turtles start depositing yolk into follicles 8–12 months prior to the nesting season (Wibbels *et al.* 1990; Miller 1997; Hamann *et al.* 2003). A pre-migratory surge of E_2 4–6 weeks prior to migration is followed by a significant and gradual decline in the concentration of this hormone 1–2 weeks before migration, declining gradually towards the end of the nesting season (Wibbels *et al.* 1990; Smelker *et al.* 2014; Myre *et al.* 2016). Although E_2 induces the production of VTG, these two biomarkers of reproduction are usually not correlated, given the long-lasting inductive effects of E_2 (Heck *et al.* 1997). Thus, E_2 peaks in advance of VTG, and VTG concentration then decreases significantly but gradually throughout the nesting season of female loggerhead sea turtles (*Caretta caretta*) (Smelker *et al.* 2014; Myre *et al.* 2016). In this way, pre-ovulatory follicles may act as a sink for VTG.

Testosterone (T) concentration in marine turtles significantly increases 1–2 weeks prior to the reproductive migration (Wibbels *et al.* 1990), and a general decreasing trend in the concentration of T has been shown for loggerhead sea turtles towards the end of the nesting season, following a trend akin that of VTG (Wibbels *et al.* 1990; Rostal *et al.* 1998; Smelker *et al.* 2014; Myre *et al.* 2016). Concentration of T is also thought to be responsible for mating behaviour (Wibbels *et al.* 1990; Hamann *et al.* 2003; Polzonetti-Magni *et al.* 2004) and for termination of the reproductive cycle (Ho *et al.* 1981; Wibbels *et al.* 1990; Myre *et al.* 2016).

The fluctuations of these biomarkers—which control ovarian recrudescence, as well as trigger sea turtle reproductive migration—are poorly understood (Hamann *et al.* 2003; Polzonetti-Magni *et al.* 2004). The objective of this study was to investigate concentration of T and VTG in green sea turtles (*Chelonia mydas*) nesting at Tortuguero, Costa Rica. We also investigated the effects of body size and condition on concentrations of reproductive biomarkers in female green sea turtles nesting at Tortuguero.

Materials and methods

Tortuguero National Park (TNP), on the Caribbean coast of Costa Rica, was created in 1970 to protect the 29 km of what is now the most important mating and nesting ground for green sea turtles in the Atlantic basin (Troëng and Rankin 2005). From 1986 to 2019, an estimated yearly average of 98 137 (± 44 633) green sea turtle nests were laid along the 29 km of Tortuguero's nesting beach (Bruno *et al.* 2020).

Green sea turtles arrive in Tortuguero in late April and early May to reproduce (Carr 1954). Nesting significantly increases in July, peaks in September and occurs through late October (Garcia-Varela *et al.* 2014). Our study was conducted in July and August 2018 on the northernmost 8 km of Tortuguero Beach, near the Sea Turtle Conservancy (STC) station.

Under Southeastern Louisiana University's IACUC and the Costa Rican Ministry of the Environment's permits (001/2018 and 002/2018, respectively), we used 18-gauge, 2.5-inch heparinized needles to collect up to 15 ml of blood from the cervical sinus of adult female green sea turtles nesting at Tortuguero. All blood sampling was conducted following Owens and Ruiz (1980) protocols. We sampled blood from nesting females during oviposition and placed the samples on ice until centrifugation, and blood plasma was stored in liquid nitrogen prior to analysis.

We exported the samples to a −80° C freezer at Southeastern Louisiana University, Hammond, LA, USA (CITES 18US75808C/9). Following procedures by Myre *et al.* 2016, we extracted steroid hormones twice from 10 to 30 µl of plasma with 5 ml of diethyl ether in each extraction. After steroid extraction, we reconstituted samples with 300 µl of assay buffer and plated it in duplicate in a 96-well microtiter plate coated with Anti-T antibodies of a commercial high sensitivity enzyme-linked immunosorbent assay (ELISA) kit (ENZO Life Sciences, Farmingdale). This assay was fully validated to measure T in green turtles (Allen *et al.*, 2015). Subsequently, we performed ELISAs according to manufacturer's specifications and read the plate in a microplate reader at 405 nm. To find the concentrations of T in each sample, ELISA results were analysed using the four-parameter logistic equation in SigmaPlot v14.0.

For the VTG assay, we followed the same protocol that was described by Smelker *et al.* (2014). For this, a solution of purified sea turtle VTG was used to create a standard curve for the indirect ELISA. We diluted the samples 1:30000 to ensure that optical density fit into the linear portion of the standard curve (between 20% and 80% of absorbance). We plated the samples in duplicate into a 96-well flat bottom polysterene microtiter plate (Thermo Fisher Scientific, Pittsburg, PA, USA), which were sealed and incubated overnight at 4°C. The next day, we washed the plates three times with 150 µl of a phosphate buffered saline (PBS) solution and a non-ionic detergent (Tween-20). To maximize specificity, we pre-incubated rabbit anti-VTG antibody with 5 µl of male sea turtle plasma in a solution of PBS with 5% non-fat dry milk (blotto) for 1 hour. Pre-adsorbed anti-VTG antibody (1:40000) was then added to the plates, which were sealed and incubated for 1 hour at room temperature on a shaking platform. After three more washes, we added 100 µl of goat

anti-rabbit antibody coupled with horseradish peroxidase diluted in blotto to the plates (BIORAD, Hercules, CA, USA) and incubated for 2 hours at room temperature on a shaking platform. After another washing cycle, we added 100 µl of tetramethybenzidine peroxidase for enzyme-immunoassay to each well of the plate and incubated for 10 minutes at room temperature to promote colour development. To stop the colorimetric reaction, we added 100 µl of sulphuric acid (1 N H_2SO_4) to each well. The plates were then read in a Bio-Rad Model 680 Microplate Reader with a 450-nm wavelength filter.

In the field, we measured curved carapace length (CCL) with a flexible, 1.5-m measuring tape on the centre of the carapace from the top of the nuchal scale to the inner part of the notch between the supracaudal scales. We measured straight carapace length (SCL) with a metal calliper between the most anterior and the most posterior part of the turtle's carapace (Wyneken 2001). Both CCL and SCL measurements were taken at least three times in cm, until three measurements did not differ by more than 1 cm. In addition to body measurements, we weighed (kg) and calculated the condition index (CI) of a sub-group of 16 female green sea turtles nesting at TNP using the following formula by Bjorndal *et al.* (2000): $\left(\mathrm{CI} = \frac{Weight}{SCL^3} \times 10^4\right)$. Finally, we marked turtles with metal tags for individual identification.

STC's research personnel has monitored sea turtle nesting activity at TNP since 1955. This included the collection of morphometric data of nesting females at the northernmost 8 km of nesting beach (Bjorndal and Carr 1989, Troëng and Rankin 2005). Since 1986, to account for the total number of nests laid at TNP during one nesting season, STC staff has conducted a weekly survey of the entire 30 km of nesting beach (Troëng and Rankin 2005; Garcia-Varela *et al.* 2014). The estimation of green sea turtle clutches laid at TNP yearly was described in detail by Troëng and Rankin (2005). For this study, we accessed STC's long-term database to extract biometric data of Tortuguero green sea turtles.

For all statistical analyses, we used SYSTAT 13.0 and we used ggplot2 package in R 3.6.3 to create the figures. To assess the normality of the data, we visually analysed the histogram of the studentized residuals and carried out One Sample KS tests. We used linear regression analyses to investigate the relationship between T and VTG and the morphometric parameters of individual female green sea turtles (such as SCL, CCL, weight and CI). To analyse temporal fluctuation of T and VTG early in the nesting season and to investigate differences in SCL and CI per month, we used analysis of variance (ANOVA).

Results

We measured circulating concentrations of T and VTG in 66 blood samples of green sea turtles nesting at Tortuguero. Two of these samples were collected from the same female and in consecutive nesting events (11 days apart). Mean T concentration was 1100 (± 1000 pg ml^{-1}). Our average intra-assay coefficient of variation (CV) for the T assays was 8% and the inter-assay CV was 12.9%. Mean VTG concentration in the samples was 27.9 (± 6.6 mg ml^{-1}). Validation and specificity testing of VTG ELISA was conducted by Smelker *et al.* (2014). Our intra-assay CV for these VTG assays was 5.1% and the inter-assay CV was 12.8%.

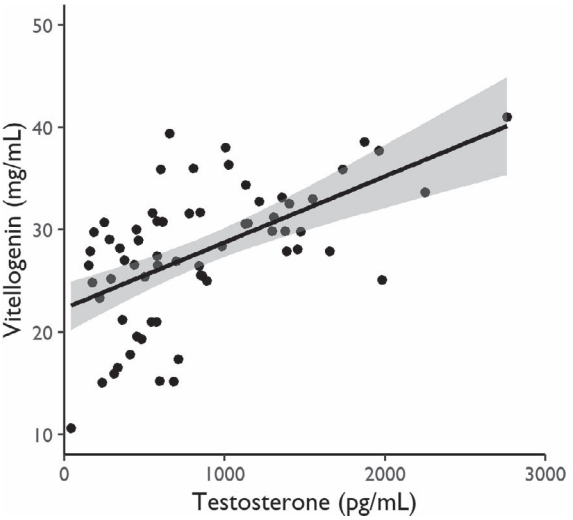

Figure 1: VTG concentration ($n = 66$) was positively correlated with testosterone concentration ($r^2 = 0.315$). Scatter plots shown with the regression line and 95% confidence interval.

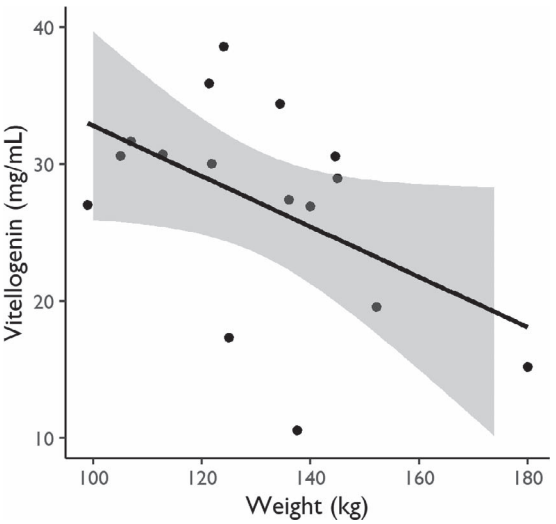

Figure 2: VTG concentration ($n = 16$) was negatively correlated with green sea turtle weight ($r^2 = 0.278$). Scatter plots shown with the regression line and 95% confidence interval.

We found a positive relationship between T and VTG (linear regression, $r^2 = 0.315$, $F_{1,63} = 28.995$, $P < 0.005$) (Fig. 1). VTG concentration was negatively correlated

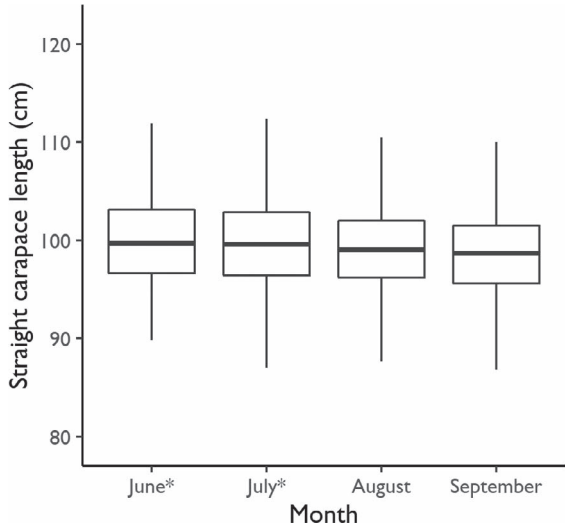

Figure 3: SCL of turtles nesting in June and July was significantly greater (asterisk) than that of turtles nesting in August and September. Boxplot shown with standard deviation and median. Whiskers showing highest and lowest observation ($n = 5547$).

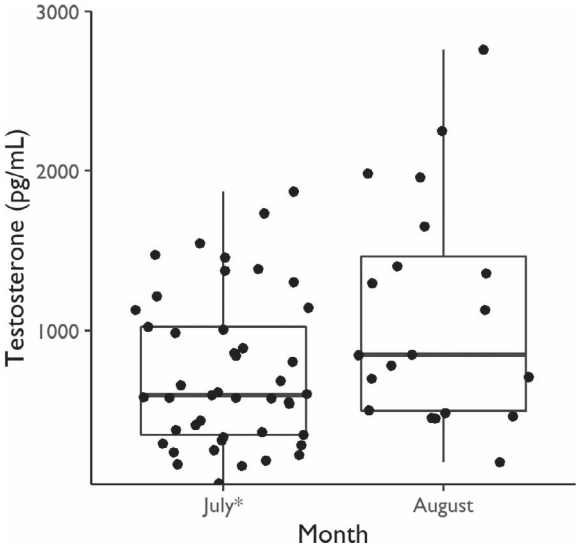

Figure 4: Testosterone concentration ($n = 66$) was significantly lower in July than in August. Boxplot shown with standard deviation, median and data points. Data points displayed with jitter to avoid overlap. Whiskers showing highest and lowest observations.

with green sea turtle weight (linear regression, $r^2 = 0.278$, $F_{1,13} = 5.017$, $P < 0.05$) (Fig. 2). SCL of female green sea turtles nesting at TNP between 1955 and 2017 varied per month, and significantly larger females nested in June and July than in August and September (ANOVA, $F_{1,5542} = 39.974$, $P > 0.001$) (Fig. 3).

Mean T concentration was significantly lower (ANOVA, $F_{1,63} = 6.426$, $P = 0.014$) in July than in August (Fig. 4). From

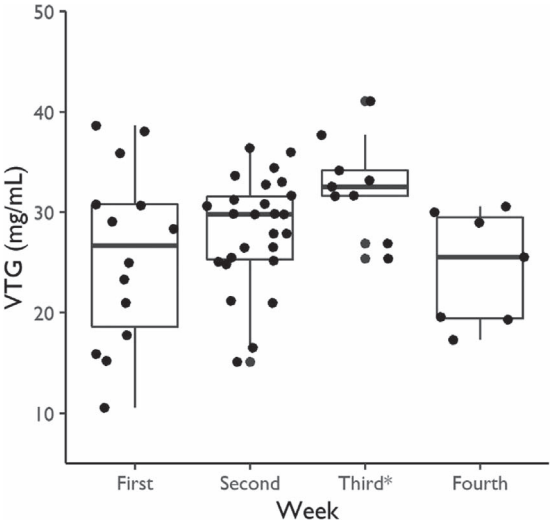

Figure 5: Concentration of VTG ($n = 52$) was significantly higher during the third week of sampling. Boxplot shown with standard deviation, median and data points. Data points displayed with jitter to avoid overlap. Whiskers showing highest and lowest observations.

20 July to 22 August, mean VTG concentration ($n = 56$) was significantly higher during the third week of sampling (ANOVA, $F_{1,52} = 7528$, $P = 0.008$) (Fig. 5). The only nesting green sea turtle we recaptured in this study showed a similar pattern of increasing T and VTG circulating concentrations over 11 days. Both VTG and T were higher in the second nesting event than in the first. However, because of the small sample size, no significance could be attributed to the change in the concentration of endocrine correlates between the two nesting events. CI was significantly lower in July than in August (ANOVA, $F_{1,14} = 7.656$, $P > 0.05$) (Fig. 6). Neither SCL, CCL nor body weight varied significantly from July to August 2018.

Finally, there was positive relationship between weight and SCL (linear regression, $r^2 = 0.613$, $F_{1,226} = 358.103$, $P < 0.005$) and CCL (linear regression, $r^2 = 0.599$, $F_{1,13} = 19.430$, $P < 0.005$) (Figs 7 and 8). However, CI was not significantly correlated to any of the other body measurements nor to the concentrations of T and VTG.

Discussion

We sampled blood from female green sea turtles during four weeks at the start of the nesting season at Tortuguero, which lasts up to 4 months. Concentration of Testosterone increased significantly from July to August, and VTG concentration, which was elevated during the whole sampling period, significantly increased during the third week of sampling. Interestingly, we sampled blood twice from the same female with an 11-day interval and observed that both T and VTG concentrations almost doubled between the first and second

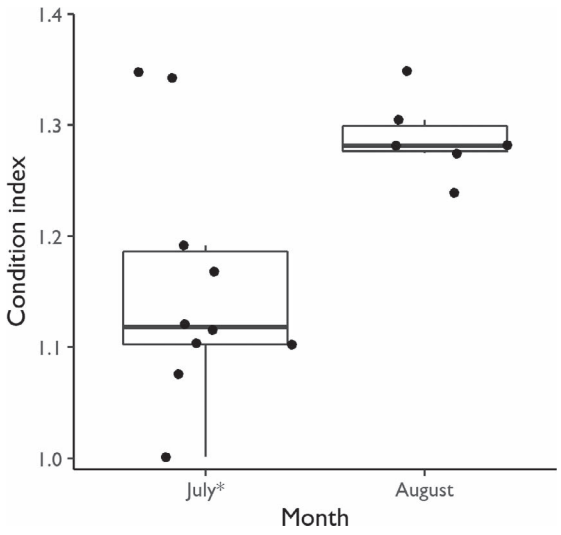

Figure 6: CI of Green sea turtles nesting at Tortuguero was significantly lower in July than in August. Boxplot shown with standard deviation, median and distribution of data points. Data points displayed with jitter to avoid overlap. Whiskers showing highest and lowest observations.

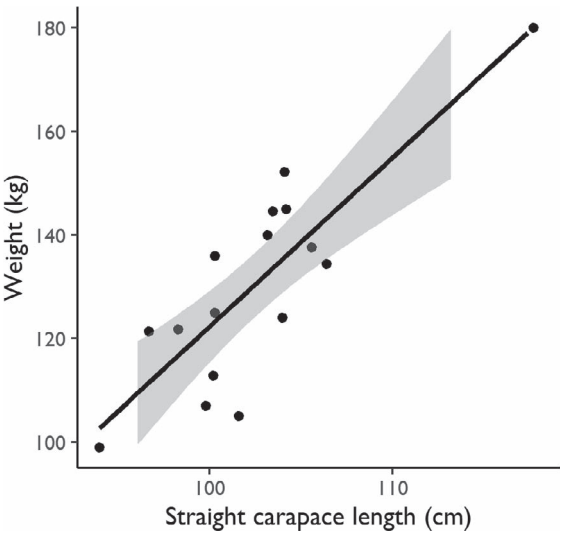

Figure 7: Green sea turtle weight ($n = 14$) was positively correlated to SCL ($r^2 = 0.613$). Scatter plots shown with the regression line and 95% confidence interval.

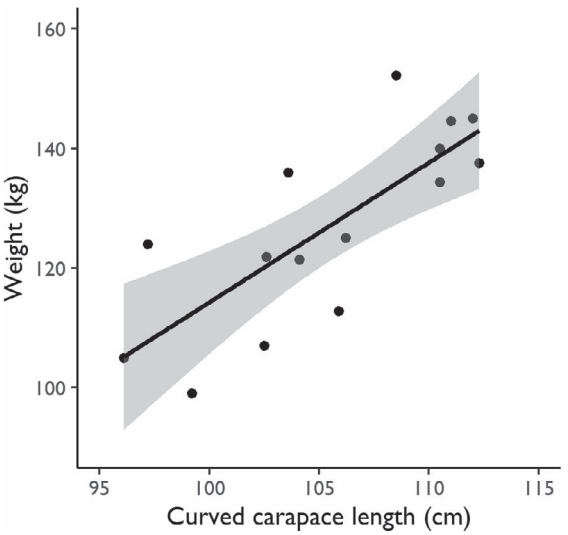

Figure 8: Green sea turtle weight ($n = 15$) was positively correlated to CCL ($r^2 = 0.599$). Scatter plots shown with the regression line and 95% confidence interval.

encounter. VTG concentration in Tortuguero green sea turtles shown in our study were up to 40 000 times greater than basal VTG concentrations in juvenile Green (Herbst *et al.* 2003) and adult female loggerhead sea turtles that were not preparing to breed (Smelker *et al.* 2014). Moreover, VTG concentration in nesting Tortuguero green sea turtles were akin to that of juvenile Green (Herbst *et al.* 2003) and Kemp's Ridley (*Lepidochelys kempii*; Heck *et al.* 1997) sea turtles injected with E_2 and of nesting Loggerheads (Smelker *et al.* 2014; Myre *et al.* 2016). As reptilian VTG estimated half-life is between 15 and 25 days (Bast and Gibson 1985), the results of our study showed that one of the components of vitellogenesis, the production of VTG (Ho 1987), was still taking place early into the nesting season of Tortuguero green sea turtles. Whether VTG produced early in the nesting season of Tortuguero green sea turtles was still being incorporated in ovarian follicles requires further research.

Elevated concentrations of gonadal steroids in loggerhead sea turtles early in the nesting season (Wibbels *et al.* 1990), the follicular hierarchy observed in necropsied nesting green sea turtles (Limpus *et al.* 2003) and pre-ovulatory surges in estradiol concentration in captive loggerhead sea turtles (Kakizoe *et al.* 2010) led authors to suggest that there may be continued follicular development early into the nesting season. In contrast, leatherback sea turtles appeared to arrive at the nesting beach with fully developed ovaries, as no hierarchy in follicular size was detected via ultrasonography (Rostal *et al.* 1996). Likewise, ultrasonography of captive Kemp's Ridleys concluded that the entire complement of ovarian follicles was fully developed by the time of mating (Rostal *et al.* 1990, 1997; Rostal 2005). However, leatherback sea turtles travel long distances from their foraging areas to their nesting grounds (Shillinger *et al.* 2008; Benson *et al.* 2011), presumably having ample time to complete ovarian development prior to arrival at nesting beaches. Kemp's Ridley sea turtles have lower clutch frequency and size than Tortuguero green turtles (Van Buskirk and Crowder 1994; Rostal *et al.* 1997; Bjorndal 1999; Rostal 2005; Shaver *et al.* 2016) and may afford the space in the coelomic cavity to hold the full complement of mature follicles for the nesting season all at once. In summary, due to distinct constraints for

reproduction of different sea turtle species and populations, timing of completion of yolk deposition into ovarian follicles may vary within the sea turtle superfamily Chelonioidea.

Concentration of VTG in our study was negatively correlated with female green sea turtle body weight, which may be due to sampling larger turtles earlier into their nesting effort, when their VTG concentrations were higher. Relationships between body size and reproductive phenology are found throughout Reptilia. For example, smaller female viviparous lizards reproduce later than their larger counterparts, presumably due to having to divide energy between growth and reproduction (Bauwens and Verheyen 1985). Additionally, larger female diamond-backed terrapins (*Malaclemys terrapin*) nest earlier than their smaller counterparts, which may allow them to have access to the fittest males in the population (Wolfe *et al*., 2021, in review). Measuring a different metric of the sea turtle reproductive output, Hatase and Tsukamoto (2008) found that the size of female loggerhead sea turtles influences reproductive frequency, with larger individuals having shorter remigration intervals than smaller ones. Larger female green sea turtles grow slower than their smaller conspecifics (Bjorndal *et al.* 2000) and may be able to reach the energetic threshold necessary for reproduction sooner and nest earlier in the season than smaller individuals. Additionally, if larger female green sea turtles swim faster, they may arrive earlier at Tortuguero than smaller females.

Our data are consistent with previous studies that have examined the morphometry of green sea turtles. Mean SCL of female Tortuguero green sea turtles was similar to values reported by Bjorndal *et al.* (2000) for adult green sea turtles throughout the Caribbean Sea. The mean CCL of our study was close to that reported for adult green sea turtles nesting in the Southern Great Barrier Reef (Limpus and Chaloupka 1997) and on Cyprus (Broderick *et al.* 2003). Green sea turtle CI in our study was similar to other populations (Bjorndal *et al.* 2000; Thomson *et al.* 2009; Labrada-Martagón *et al.* 2010). Moreover, in agreement with Bjorndal *et al.* (2000), CI was not significantly correlated with SCL, CCL or weight in our study. Mean body weight was unsurprisingly strongly correlated to green sea turtle SCL and CCL. From the standpoint of morphometry, our results suggest that our findings with reproductive biomarkers of gonadal function may be applicable to other green sea turtle populations.

Mean concentration of VTG was higher and mean concentration of T was lower than previously reported for loggerhead sea turtles (Wibbels *et al.* 1990; Smelker *et al.* 2014; Myre *et al.* 2016). Owens (1980) and Wibbels *et al.* (1990) reported that green sea turtles have an overall lower concentration of gonadal steroids than other sea turtle species, and dietary intake has previously been cited to affect concentrations of gonadal steroids and overall reproductive output in female reptiles (Limpus and Nicholls 1988; Chaloupka *et al.* 2008; Lovern and Adams 2008). Finally, both Wibbels *et al.* (1990) and Smelker *et al.* (2014) used a radioimmunoassay to measure T in their samples, whereas we used ELISAs, which complicate direct comparisons. Radioimmunoassay and ELISA have been reported to yield slightly different, but correlated results when used to measure concentration of gonadal and adrenal steroids (Lewis *et al.* 1986; Khatun *et al.* 2009).

Understanding the reproductive physiology of endangered species is crucial for conservation, and ours was the first study investigating reproductive biomarkers of endangered green sea turtles at their most important breeding ground in the Atlantic Basin. The information provided here can be used to guide further studies regarding the fluctuation of reproductive biomarkers during the nesting cycle and the timing of completion of yolk deposition into the ovarian follicles of free-ranging sea turtles.

Funding

This work was supported by the National Geographic Society [EC-51596-18], Sea Turtle Conservancy, Sigma Xi Grant in Aid of Research, American Museum of Natural History, IDEAWILD, CORBANA and Varcli Pinares S.A. Kenneth (Major) Dyson Professorship to R.A.V.

Conflict of interest

The authors declare that they have no conflict of interest.

Acknowledgements

The authors thank the community and national park rangers of Tortuguero, Costa Rica, for their support. The author thanks all STC research assistants and staff and all the personnel from ASVO Tortuguero and ASOPROTOMA.

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