Ovarian Dynamics in Free-Ranging Loggerhead Sea Turtles (*Caretta caretta*)

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Vitellogenin (VTG) is an egg yolk-precursor protein that serves as a nutrient source for developing embryos in oviparous vertebrates. The hormonal control of this protein has been studied in a variety of taxa, but details about the dynamics of this protein remain to be elucidated in sea turtle species. To investigate the dynamics of VTG in a multi-clutch species under natural conditions, 38 adult Loggerhead females entrained in the Florida Power and Light St. Lucie Nuclear Plant intake canal in Hutchinson Island, Florida were sampled from May–August of 2014. Blood samples were drawn to measure testosterone, estradiol 17β, and vitellogenin (T, E_2, and VTG, respectively) using enzyme-linked immunosorbent assays. Ultrasound imaging of the gonads was used to determine ovarian status and to measure ovarian follicle and oviductal egg size. Results showed that VTG concentration increased from May (8.27 mg mL^{-1}) to June (15.37 mg mL^{-1}) and declined into July and August (9.44 mg mL^{-1}); this decline corresponded with the end of the nesting season. E_2 declined from 718.02 pg mL^{-1} in May to 95.89 pg mL^{-1} in July–August, and T declined from 2,008.35 pg mL^{-1} in May to 1,221.24 pg mL^{-1} in July–August. Mean concentration for both gonadal steroids was significantly higher in reproductively active females than means of reproductively inactive females, though overlapping concentrations of the steroids occurred between active and inactive animals. However, VTG concentration was high in reproductively active turtles and undetectable in gonadally quiescent turtles. We concluded that the addition of VTG measurement in conjunction with the gonadal steroids provides a more accurate and easily interpretable way to predict reproductive status of adult Loggerhead females. Finally, gonadal steroid and VTG concentration in our study corresponded only with late nesting animals, indicating that early season females do not become entrained in the intake canal of the power plant.

SEA turtles are unrivaled among reptiles for their capacity to produce massive numbers of eggs during a reproductive season (Wallace et al., 2007). Owens (1980) emphasized the importance of gaining a better understanding of sea turtle reproductive physiology due to the conservation status of these species as threatened or endangered. In the past 30 years, physiologists have made significant strides in better understanding the roles of gonadotropins and gonadal steroids during breeding, especially in female reptiles. Despite these advancements, gaps in our understanding of vitellogenesis and its regulating factors persist (Hamann et al., 2003), particularly in sea turtle species. Questions remain, especially surrounding vitellogenesis and follicular growth during gonadal recrudescence (Licht, 1982). Hamann et al. (2003) called for studies targeting seasonal vitellogenin (VTG) production, and the present study contributes supplementary data to studies in nesting Loggerhead females (Wibbels et al., 1990; Whittier et al., 1997; Smelker et al., 2014) and in captive sea turtles (Sifuentes-Romero et al., 2006; Kakizoe et al., 2010). We aimed to address the gaps in our understanding of VTG production by describing VTG and the dynamics of gonadal steroids estradiol-17β (E_2) and testosterone (T) in wild Loggerhead females in the ovarian cycle (i.e., recrudescence and regression) over a reproductive season. Here we provide data on the reproductive physiology of in-water turtles to elucidate VTG dynamics during gonadal regression and demonstrate the need for additional research of the reproductive physiology of sea turtles.

The production of VTG prepares the female reproductive tract for the formation of egg yolk (Ho et al., 1982). Ovarian growth is dependent upon the uptake of VTG (Callard and Ho, 1987). VTG is an egg yolk-precursor protein that is synthesized in the liver and incorporated into ovarian follicles as phosvitins and lipovitellins, which serve as a nutrient source for developing embryos (Ho et al., 1982; Wallace, 1985). Understanding the cyclical and seasonal patterns of vitellogenesis is crucial for threatened species, as vitellogenin represents an essential nutritional investment of female sea turtles to ensure the success of their offspring.

Ovarian growth has been documented to begin as early as 8–10 months prior to migration in sea turtles (Wibbels et al., 1990; Rostal et al., 1997). However, no studies have measured VTG prior to the reproductive season. VTG induction by E_2 has been described in several oviparous species such as fish (Kime et al., 1999), amphibians (Baker and Shapiro, 1977), and reptiles (Brasfield et al., 2002). Of particular interest are studies that have demonstrated E_2 induction of VTG in freshwater turtles (Ho et al., 1982; Palmer and Palmer, 1995; Tada et al., 2003) and in sea turtles (Owens, 1974; Heck et al., 1997; Herbst et al., 2003; Sifuentes-Romero et al., 2006). Loggerhead sea turtles experience a surge in E_2 immediately prior to reproductive migration (Wibbels et al., 1990). This E_2 surge coincides with ovarian growth and declines after the nesting season (Wibbels et al., 1990; Kakizoe et al., 2010).

On the Florida coast, the Loggerhead sea turtle egg-laying season begins in April and continues through mid-September. Females lay between one and seven clutches each reproductive year and are thought to exhibit a multiannual cycle, typically nesting every two to four years (Bjorndal et al., 1983; Wibbels et al., 1990; Ehrhart et al., 2014). The conservation status of the Loggerhead sea turtle motivates further understanding of its reproductive physiology. The International Union for the Conservation of Nature (IUCN) has divided Loggerhead sea turtle populations into nine distinct segments. Northwest Atlantic Loggerheads, including those that nest in the Southeastern United States, are currently classified as threatened, though Loggerheads in

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other parts of the world are listed as endangered (USFWS and NMFS, 2011). These turtles comprise approximately 90% of the reproductive effort in the Southeastern US basin (Encalada et al., 1998; Ehrhart et al., 2014) and may have more nesting Loggerheads than any other population in the world (Witherington et al., 2006). The Florida Power and Light St. Lucie Nuclear Plant on Hutchinson Island, Florida (SLNP) is located adjacent to the core Loggerhead nesting population in Florida. This power plant pulls in sea water as part of the coolant system of the nuclear reactors and routinely entrains sea turtles, from juveniles to adults. Inwater Research Group (unpubl.) reported that Loggerheads are the most commonly entrained sea turtle species at the power plant, and >90% of adult Loggerheads captured are caught between April and August. Of the adult Loggerheads entrained, a large majority (>90%) are females, which is likely due to its proximity to one of the densest Loggerhead nesting beaches in Florida (Witherington et al., 2006). A specific enzyme-linked immunosorbent assay (ELISA) has been used to describe nesting Loggerhead VTG concentration (Smelker et al., 2014). We used the same ELISA to further investigate circulating VTG concentration in free-ranging adult female Loggerheads at the SLNP.

In this study we describe gonadal steroid and VTG dynamics in Loggerhead females during specific stages (i.e., growth of ovarian follicles, presence of oviductal eggs, and ovarian regression) of the ovarian cycle over the nesting season. Additionally, we described trends in gonadal steroids and VTG in conjunction with ovarian follicle and egg size. We expected to obtain a random sample of adult females in various stages of gonadal activity, from recrudescing to gonadally quiescent animals. We hypothesized that seasonal VTG and gonadal steroid concentration would parallel the significant monthly decline previously described (Licht et al., 1980; Wibbels et al., 1990, 1992; Kakizoe et al., 2010; Smelker et al., 2014). We expected a strong, positive correlation between the gonadal steroids, VTG, and follicle size. As the season progressed, we expected a higher incidence of atretic follicles, and differing VTG concentrations in turtles that exhibit atresia.

MATERIALS AND METHODS

Study site.—The individuals used in this study were captured in the intake canal of SLNP between May–August, 2014. The SLNP is located approximately halfway between Ft. Pierce Inlet and St. Lucie Inlet, Florida. The SLNP circulates sea water through a long intake canal as part of the coolant system for the nuclear reactors. All five species (*Caretta caretta*, *Chelonia mydas*, *Dermochelys coriacea*, *Eretmochelys imbricata,* and *Lepidochelys kempii*) known to commonly inhabit or migrate through the North Atlantic are entrained in the intake canal of the power plant throughout the year. Approximately 80% of the annual 53,000 to 92,000 nests in the United States occur in six Florida counties, including St. Lucie County where the SLNP is located (Witherington et al., 2006). The power plant pulls sea water through three large diameter pipes (3.7–4.9 m) and circulates it around the nuclear reactors as part of the cooling system of the plant. The pipe openings are perpendicular to the sea floor and are contained within intake structures approximately 365 m offshore, 3 m above the ocean floor in 7 m of water. The intake pipes are protected by large velocity caps that sit 2 m above the pipe openings, and transport water at a rate of approximately one million gallons per minute. Sea turtles that travel into the pipe openings are rapidly transported into a 1500 m intake canal where they are captured via tangle net, dip net, or by hand. Sea turtles entrained in the power plant intake system are confined to the easternmost portion of the intake canal by permanent barrier nets which prevent travel further west into the plant.

To further investigate the reproductive physiology of Loggerhead sea turtles, we used the SLNP as a source of random, reproductively active Loggerhead females. We collected blood samples to measure T, E_2, and VTG in 38 Loggerheads captured in the intake canal of the power plant during the summer of 2014. We divided Loggerhead females into groups based on the months they were sampled to look at monthly trends. We determined sexual maturity and reproductive status using body measurements and gonadal ultrasound.

Sample collection.—We collected samples from all adult Loggerhead sea turtles $\geq$ 80 cm straight carapace length (SCL) from 15 May 2014 to 3 August 2014. This period corresponded with the height of the nesting season for Loggerheads in Florida and allowed us to sample from 86% of adult Loggerheads entrained in the intake canal of the power plant in 2014. The 80 cm SCL minimum measurement to classify adult status is reasonably conservative given that female Loggerheads nesting on Hutchinson Island have carapace lengths that range as low as 71.1 cm (Hirth, 1980). This measurement was also used to determine adult size when studying the sex ratios of immature Loggerhead sea turtles in Florida, including turtles at the SLNP (Wibbels et al., 1987). In that study, adult status was reliably determined by tail length when SCL > 80 cm.

Once a turtle was removed from the intake canal, we disinfected the area around the dorsal cervical sinus with betadine and isopropyl alcohol and used a 21-gauge, 1.5 inch heparinized needle to collect 10–15 cc of blood from each animal, as previously described (Owens and Ruiz, 1980). We noted the time from capture to blood collection. We placed the whole blood immediately on ice and centrifuged it within 1 hour of collection to separate the plasma, which we stored in liquid nitrogen. We transported samples on dry ice to Southeastern Louisiana University and stored them at –80°C until analysis. We weighed and measured all turtles entrained in the intake canal; we also tagged each animal with passive integrated transponder (PIT) tags and Inconel tags.

Prior to release, we placed turtles in dorsal recumbency on a padded surface for ultrasound examination of the gonads using a Sonosite TITAN ultrasound instrument (Sonosite, Inc., Bothell, WA) with an 8-5 MHz curved transducer. We classified females according to their ovarian status as per ultrasonograms: we classified turtles with vitellogenic ovarian follicles but no shelled eggs in the oviduct as "follicles only" (F), females with vitellogenic ovarian follicles and shelled eggs present in the oviduct as having "eggs and follicles" (EF), females with ovarian follicles, shelled eggs in the oviduct, and follicles showing atresia as having "eggs, follicles, and atretic follicles" (EFAF), females with ovarian follicles and atretic follicles as "follicles and atretic follicles" (FAF), and females with no evidence of developing follicles or eggs in the oviduct as "neither eggs nor follicles" (NENF). We used electronic calipers on the Sonosite to measure diameter (cm) of ovarian follicles, egg yolk, and eggshells of shelled eggs in the oviduct. Following examination, we released

turtles immediately north of the power plant on the adjacent beach.

Plasma hormone concentration.—We measured circulating E_2 concentration with a commercial ELISA kit (Cayman Chemical, Ann Arbor, MI). We added between 6–1,000 μL of plasma (we extracted most samples with diethyl ether at 100 μL of sample) to a glass test tube with enough enzyme immunoassay (EIA) buffer to equalize sample volumes before extraction. We performed a double extraction with 2X the sample volume of diethyl ether each for each extraction. After vortexing, we flash froze the aqueous phase of each sample in a dry ice and ethanol slurry and decanted the ether fraction into a fresh test tube. We then evaporated the ether under a gentle stream of N_2 gas in a 37°C water bath. We reconstituted each sample with 175 μL EIA buffer and ran each sample in triplicate per the manufacturer's instructions. We read plates using a Bio-Rad model 550 microplate reader at 415 nm. We corrected predicted E_2 concentrations for sample volume extracted prior to statistical analysis. A parallelism test with a sample pool showed that the assay was appropriate for measuring E_2 in Loggerheads.

We measured T concentration using a commercial kit (Enzo Life Sciences, Farmingdale) as per the manufacturer's specifications. We added between 15–1,000 μL of plasma (we extracted most samples with diethyl ether at 30 μL of sample) to a clean test tube with enough EIA buffer to equalize sample volumes and extracted 2X with diethyl ether as described above. We reconstituted with 250 μL EIA buffer and ran each sample in duplicate. We read plates using a Power Wave HT Spectrophotometer at 405 nm. We corrected predicted T concentrations for sample volume extracted prior to statistical analysis. A parallelism test with a sample pool showed that the assay was appropriate for measuring T in Loggerheads.

Vitellogenin concentration.—We conducted the VTG ELISA as previously described (Smelker et al., 2014). Briefly, we diluted standards and samples using phosphate-buffered saline (PBS) at dilutions ranging from pure plasma to a 1:7,500 dilution, then plated onto 96-well microtiter plates and incubated at 4°C overnight. We washed plates with PBS and blocked with PBS Blotto (5 g nonfat dry milk per 100 mL PBS) and allowed the plates to incubate on a gentle shaker for 2 h. To ensure maximum specificity of the antibody, we diluted the anti-Loggerhead VTG antibody 1:1,000 in PBS Blotto and presorbed the antibody at 4°C for 1 hour with 3 μL plasma of juvenile *Caretta caretta* that was run on a previous assay and determined to have an undetectable VTG concentration. We diluted the presorbed primary antibody 1:40,000 prior to plating. We washed the plate, coated it with primary antibody, and allowed it to incubate for 2 h with gentle shaking. After washing, we used a diluted (1:2,000) goat anti-rabbit IgG coupled to horseradish peroxidase (Bio-Rad Laboratories Inc.) to coat each well and incubated for 2 h. After washing, we developed the plate by adding 100 μL TMB Peroxidase EIA Substrate (Bio-Rad Laboratories Inc.) to each well. We stopped the reaction using 1 N H_2SO_4 and read the plate using a Bio-Rad Model 550 microplate reader at 450 nm. The VTG standard curve generated for this assay had a working range of 0.08 μg mL^{-1} to 40 μg mL^{-1} with a detection limit of 1.1 μg mL^{-1}. We estimated the detection limit for this assay to be two standard deviations from the mean blank absorbance readings for each plate ($n = 2$).

Statistical analyses.—We used correlation analysis to test hypotheses about the relationships of E_2, VTG, and T to SCL, follicle size, yolk size, and egg shell size, and their relationships to each other. Prior to correlation analysis we log transformed data for E_2, VTG, and T. We used analyses of variance (ANOVA) to test hypotheses of how VTG, the gonadal hormones, and follicle size varied over the sampling period and across ultrasound category (i.e., F, EF, EFAF, NENF); the FAF category was eliminated from all ANOVA analyses because of low sample size ($n = 1$). For all ANOVAs, we replaced two missing cells (turtles with only follicles in July and August, and turtles with eggs, follicles, and atretic follicles in May) with means of VTG, E_2, T, or follicle size±1 standard deviation for the respective ultrasound category. We performed Kruskal-Wallis tests to determine normality of data, and we performed Levene's tests to determine homogeneity of variance. Due to unequal variances, we performed a non-parametric ANOVA on E_2. To meet the assumption of normality, data for T was log transformed for ANOVA analysis. We considered $P < 0.05$ significant in all analyses. We used Tukey tests or contrasts for *post-hoc* analyses. All statistics were performed using SigmaPlot 11.0 (SigmaPlot Software, Inc.) and SYSTAT 13.0 (Systat Software, Inc.). We used SigmaPlot 11.0 to create figures. Statistics are reported as the mean±standard error (SE).

RESULTS

We recovered a total of 38 adult Loggerheads from the intake canal. Blood samples were taken from all adult females entrained in the SLNP during the peak reproductive season ($n = 38$). Average SCL was 87.0±5.3 cm and average mass was 97.3±21.0 kg. All but one turtle were also examined via ultrasound and categorized as F ($n = 4$), EF ($n = 16$), FAF ($n = 1$), EFAF ($n = 11$), and NENF ($n = 5$). Examples of each of these structures are depicted in Figure 1. Turtles were classified according to the month they were captured prior to analysis (May: $n = 7$; June: $n = 18$; and July–August: $n = 13$).

The average E_2 intra-assay CV was 7.4% and the inter-assay CV was 14.2%. For T, the average intra-assay CV was 10.3% and inter-assay CV was 9.5%, and the average intra-assay CV for VTG was 4.5% and inter-assay CV was 21.4%.

A separate one-way ANOVA was performed for VTG, E_2, T, and follicle size across ultrasound category and sampling period. Ultrasound categories for the gonadal hormone ANOVA included F, EF, EFAF, and NENF. For the VTG ANOVA, NENF females were eliminated from the analysis because the VTG concentrations were below the detection limit of the assay.

Vitellogenin.—We measured VTG concentration in all but five females sampled, and VTG concentration ranged from 1.80–23.17 mg mL^{-1}. Correlation analysis determined that VTG, T, and E_2 all significantly and positively correlated with each other. VTG and T ($r = 0.360$, $P = 0.036$) as well as VTG and E_2 were significantly and positively correlated ($r = 0.376$, $P = 0.028$). T and E_2 were also positively correlated ($r = 0.707$, $P < 0.001$). Follicle, egg yolk, and egg shell size were not significantly correlated with the steroids, VTG, SCL, mass, body width, or tail length. Body measurements and T, E_2, VTG, SCL, and mass were also not significantly correlated.

ANOVA statistics showed VTG concentration varied significantly across sampling month ($F_{2,31} = 1.25$; $P < 0.05$). Tukey tests showed that VTG significantly increased from May to June and significantly decreased into July and August (Fig. 2).

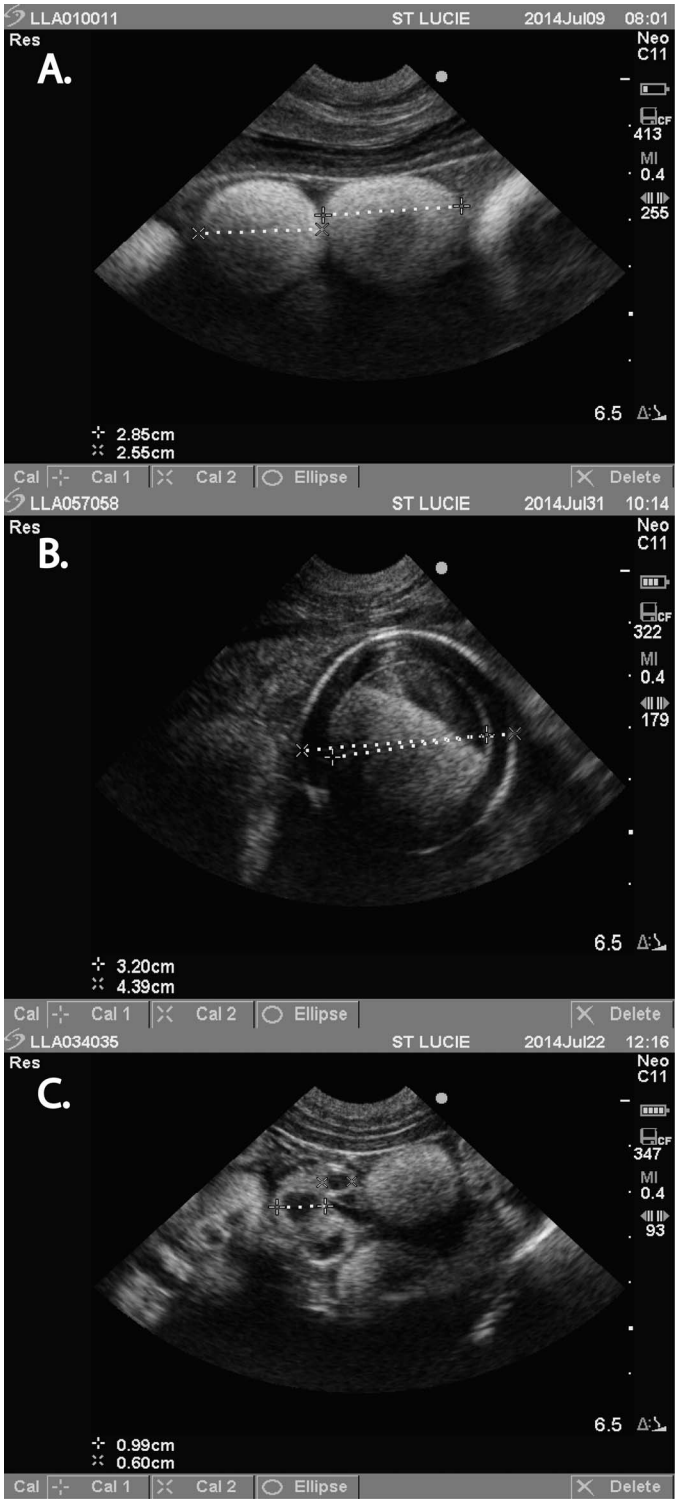

Fig. 1. Gonadal ultrasound images generated with a Sonosite TITAN ultrasound instrument with an 8-5 MHz curved transducer. Images depict examples of: (A) developing vitellogenic follicles, (B) shelled eggs in the oviduct, and (C) atretic follicles in Loggerhead sea turtles captured at Florida Power and Light St. Lucie Nuclear Plant, Florida. Structures of interest are measured.

We observed no turtles with atretic follicles until mid-June, and we observed atretic follicles in only four out of 18 females sampled in June. However, in turtles sampled during July to the beginning of August, we found seven out of 13 turtles had atretic follicles. VTG concentrations varied significantly across ultrasound category ($F_{2,31} = 3.87$; $P < 0.05$). *Post-hoc* contrast analysis showed VTG was significantly highest in turtles with EFAF compared to F or EF turtles (Fig. 3).

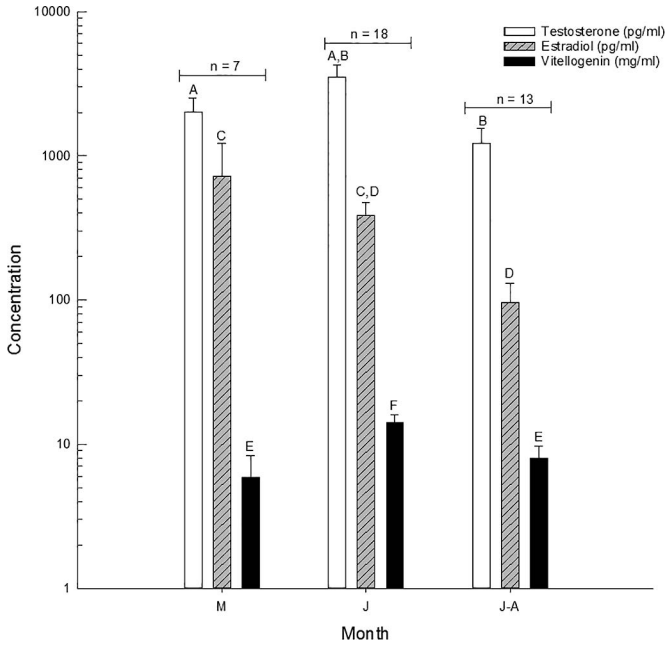

Fig. 2. Mean T, E_2, and VTG concentration in Loggerhead sea turtles captured at the Florida Power and Light St. Lucie Nuclear Plant, Florida over the study period (M = May, J = June, J-A = July–August). VTG concentration is significantly higher in June than in May or July–August. T and E_2 are significantly lower in July–August than in May. Letters above bars indicate Tukey test significant differences.

Five turtles sampled throughout the season (average SCL: 81.0±1.12 cm; average mass: 77.36±9.51 kg) showed no evidence of ovarian activity as per the ultrasound and were classified as NENF. All turtles in this study were classified as adults via SCL > 80 cm. A previous study on Loggerhead sea turtle sex ratios classified adults > 80 cm SCL as adult males at tail lengths ≥ 400 mm and adult females at tail lengths ≤ 250 mm, with tail lengths > 250 mm and < 400 mm classified as unknown (Wibbels et al., 1987). The lack of tail elongation in the NENF group (average tail length: 200.0±44.95 mm) indicated that these individuals were reproductively inactive females, and not males. All individuals in the NENF group had T concentrations (average: 430 pg mL^{-1}) that fell within the range of T measured (15 pg mL^{-1} to 1209 pg mL^{-1}) in adult females in the same population. All NENF turtles had undetectable VTG concentration.

Gonadal steroids.—T and E_2 concentrations were detectable in all females sampled ($n = 38$). T ranged from 50–12,900 pg mL^{-1} and E_2 ranged from 3.8–3,723 pg mL^{-1}. T and E_2 showed significant differences across sampling month (T: $F_{2,36} = 3.52$, $P < 0.05$; E_2: $F_{2,36} = 12.57$, $P < 0.001$). T and E_2 followed seasonal trends similar to one another, which showed a slight, non-significant decrease from May to June, and Tukey tests determined that this decrease was significant in turtles sampled in July and August (Fig. 2).

The mean T and E_2 concentrations also showed significant differences across ultrasound category (T: $F_{3,36} = 7.24$, $P < 0.001$; E_2: $F_{3,36} = 7.58$, $P < 0.001$), and gonadal steroids were significantly lower in NENF turtles but were not significantly different among the other ultrasound categories (Fig. 3). One

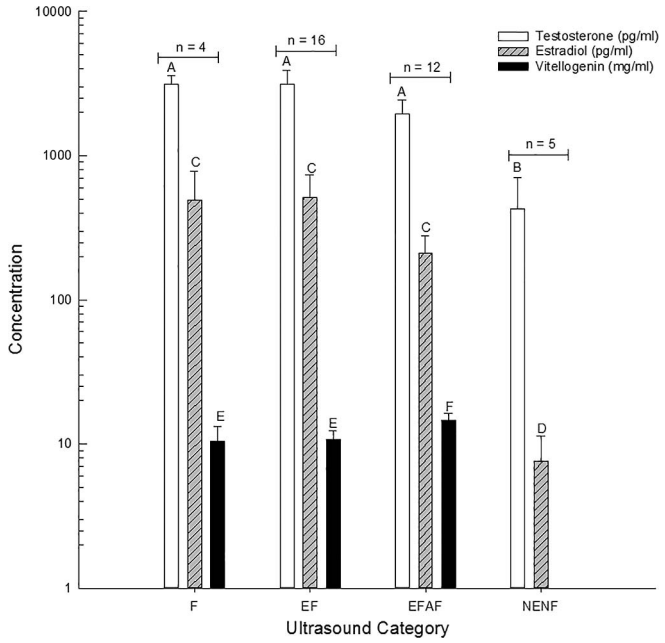

Fig. 3. Mean T, E_2, and VTG concentration across ultrasound category (F = follicles only; EF = oviductal eggs and follicles; EFAF = eggs, follicles, and atretic follicles; NENF = neither eggs nor follicles) in Loggerhead sea turtles captured at the Florida Power and Light St. Lucie Nuclear Plant, Florida. VTG is significantly higher in EFAF turtles than in any other category. T and E_2 concentrations were lower in NENF turtles than in any other ultrasound category. Letters indicate significant contrast differences.

turtle was found to have follicles and atretic follicles with no shelled eggs detectable by ultrasound examination. This turtle was not included in ultrasound ANOVAs. T concentration in this individual was higher than the mean for any other ultrasound category (6,700 pg mL^{-1}), while E_2 and VTG concentrations were similar to the means for EFAF turtles (E_2: 118.7 pg mL^{-1}; VTG: 19.9 mg mL^{-1}). Follicle size did not vary significantly over the sampling period or by ultrasound category.

VTG was undetectable in the reproductively quiescent females sampled (NENF). The mean T (429.96±249.83 pg mL^{-1}) and E_2 (7.54±3.84 pg mL^{-1}) concentrations for the NENF category were significantly lower than those found in reproductively active females (F, EF, and EFAF). However, two individuals out of five in the NENF category had a T concentration that overlapped with concentrations seen in reproductively active females (T: 600 pg mL^{-1} and 1,500 pg mL^{-1}), and three NENF individuals out of five had E_2 concentrations that overlapped with concentrations seen in reproductively active females (E_2: 4.8, 4.9, and 22.8 pg mL^{-1}). Though digestive organs were not being investigated in this study, four out of five NENF turtles also had evidence of contents within the intestine. Interestingly, two of 33 reproductively active females also had contents within the intestine.

DISCUSSION

In this study we describe VTG and gonadal steroid dynamics in free-ranging Loggerhead female sea turtles captured in the water over a reproductive season. Circulating VTG concentrations for wild, adult Loggerhead sea turtles before or after the nesting season are not currently known. We originally expected that the Loggerheads entrained in the SLNP would provide a representative sample of Loggerhead females in various stages of ovarian activity (i.e., recrudescence and regression). However, our VTG and T concentrations were lower than expected based on previous studies and were consistent with females that were in the latter stages of their individual reproductive season. Indeed, Smelker et al. (2014) reported VTG and T concentrations from nesting individuals throughout the peak nesting season in the same area of our study. They found that females nesting during the second half of July averaged a VTG concentration of 12 mg mL^{-1}, similar to those measured across all months in our study, and a mean T concentration of about 14,000 pg mL^{-1} in the first half of June, which is three times the largest monthly average reported in the present study (May: 2,010 pg mL^{-1}; June: 3,830 pg mL^{-1}; July–August: 1,220 pg mL^{-1}). However, in another study of nesting female Loggerheads in Australia, T concentrations showed a similar progressive decline, but the highest concentration measured was 600 pg mL^{-1}, lower than the concentrations reported in the present study (Whittier et al., 1997). It is likely that these differences are due to differences in the assays used. Taken together, these data suggest that among the reproductively active turtles, the SLNP entrains only Loggerheads that are at the end of the individual reproductive season.

The reasons why no early season nesting females were entrained in the SLNP intake canal are unknown. It is possible that females entered the intake pipe looking for shelter, as Loggerheads are often found nestled in the substrate of the area surrounding the intake pipes of the SLNP. Alternatively, Loggerheads may have followed a prey item into the intake pipe as a result of reinitiating feeding. The area surrounding the intake structures of the power plant include hard bottom substrates and sabellarid worm reefs, and this area has been described as a potential foraging area for sea turtles (Martin and Ernst, 2000). The presence of a velocity cap over the intake pipes of the SLNP prevents sea turtles from entering involuntarily as they pass the pipe; instead they must swim under the cap in order to be pulled by the current into the pipe. Early season females are not likely to be actively looking for prey due to high E_2 concentration, as even low concentration of E_2 has been shown to inhibit feeding and growth in female sea turtles (Owens, 1974, 1976). As E_2 concentration declines across the season (Wibbels et al., 1990; Kakizoe et al., 2010), females may begin to feed once concentration is sufficiently low. The mechanism that reinitiates feeding in sea turtles is not currently well understood. In birds, feeding behavior is regulated by a genetically determined energy threshold, and once body condition drops below this critical threshold, refeeding is initiated (Cherel et al., 1988; Gauthier-Clerc et al., 2001; Hamann et al., 2003). It is possible that a similar mechanism may be used by sea turtles, as corticosterone and plasma triglyceride concentrations have been shown to have a positive relationship (Hamann et al., 2002a). However, a surge in corticosterone in sea turtles at the end of the season has not been reported (Whittier et al., 1997; Rostal et al., 2001; Hamann et al., 2002b), suggesting that the mechanism that initiates refeeding may be more subtle because sea turtles do not drop below a critical body condition threshold.

Gravid *Chelonia mydas* have been documented feeding (Tucker and Read, 2001), and a negligible amount of food has been found in the intestines of nesting Loggerhead females compared to nonnesting females in the same habitat (Limpus et al., 2001). Interestingly, two females examined via ultrasound in our study had evidence of oviductal eggs

and/or active ovaries, as well as contents within the intestine, and four out of five turtles with no eggs or follicles detected on the ultrasound had contents in the intestine. This suggests that some female Loggerheads sampled in our study ingested prey to some extent during the latter part of their reproductive season. Bjorndal et al. (1983) described post-nesting migrations in Loggerheads nesting on Melbourne Beach, Florida and found that post-nesting Loggerheads may travel northward, southward, or some combination of the two. Thus, near-post-nesting or post-nesting Loggerhead females may be traveling past the SLNP on their way back to their feeding grounds.

In this study we examined statistically the relationship of blood parameters with follicle size of the turtles entrained in the SNLP. Our correlation analyses failed to detect a significant relationship between VTG, T, or E_2 with follicle size. We feel that this is likely due to follicle size remaining constant in all the individuals studied. However, we observed a strong positive correlation between VTG and E_2 concentrations. This was expected given that E_2 is the endogenous inducer of VTG production. VTG induction by exogenous E_2 has been demonstrated in several sea turtle species (Owens, 1974; Heck et al., 1997; Herbst et al., 2003; Sifuentes-Romero et al., 2006). In many squamates, the majority of vitellogenesis takes place in a brief period just before mating and the first ovulation; however, freshwater turtles exhibit a more extended period of vitellogenesis (Licht, 1982). Protracted vitellogenesis seems unlikely in sea turtles, given their extended quiescent periods and the high metabolic costs of maintaining active ovaries (Wallace et al., 2005) during nonnesting years. VTG and T were also positively correlated with each other; as VTG facilitates the growth of the follicle and as the follicle grows, T is produced in higher concentrations (Owens, 1980). This same correlation was exhibited by Loggerheads in the same area in a previous study (Smelker et al., 2014). The strong, positive correlation that we observed between T and E_2 was expected in late season turtles. This correlation was also exhibited by captive (Kakizoe et al., 2010) and wild (Wibbels et al., 1990) Loggerheads, with both T and E_2 reaching near basal concentration following the final nesting event. Serial sampling of captive Loggerhead sea turtles also supports this close association, showing that a surge in T occurs immediately before a surge in E_2 surrounding nesting events (Kakizoe et al., 2010). The results of that study suggested that T is used as a precursor of E_2, but this relationship appears to be dependent on the concentration of T. In freshwater turtle species, T has been postulated to have an inhibitory role on vitellogenesis, and these inhibitory effects of T on VTG production were most effective at low concentrations; at high concentrations, the inhibitory effects of T on VTG production were reversed (Ho et al., 1982). However, it is not known if T has any inhibitory effects on VTG production in sea turtles (Ho et al., 1982).

We originally hypothesized that seasonal VTG concentration would parallel the significant monthly concentration decline in gonadal steroids previously described in various studies (Ho et al., 1982; Wibbels et al., 1990; Heck et al., 1997; Smelker et al., 2014). Though VTG did not show the expected decline, as this protein exhibited a peak in June, we observed a decrease in E_2 and T as the season progressed. These results were expected as E_2 is the main driver of VTG production (Ho et al., 1982; Heck et al., 1997), and so E_2 is expected to be at its highest concentration prior to or at the beginning of the season. Also, T is expected to gradually decline through the season, as T concentration is dependent upon follicular cells, a fraction of which ceases to produce T after each ovulation (Owens, 1980).

Our results showed that plasma VTG concentration exhibited a peak in June, significantly higher than in Loggerheads sampled in May and in July–August. This suggests that VTG production is still ongoing at this time, as has been previously proposed (Kakizoe et al., 2010). However, vitellogenesis has been reported to be complete in sea turtles before the first clutch of the season is laid (Miller and Limpus, 2003). We feel that our June peak in VTG represents a sampling artifact, since the animals included in the current study represent an assortment of individuals in various reproductive stages, which happen to be skewed toward the end of the individuals' reproductive season. Significant monthly declines in T and E_2 have been reported in Loggerheads, and so it was surprising that the decrease in gonadal steroids from May to June would be nonsignificant. This may be further evidence of a sampling artifact in the turtles that we sampled at SLNP, as the mean gonadal steroids' concentrations for the June turtles were higher than expected.

In this study we also investigated ovarian condition directly via ultrasonography. Five females had no evidence of ovarian activity on the ultrasound and we categorized them as NENF (average SCL: 81±1.12 cm; average mass: 77±9.51 kg). The five NENF females sampled in this study had undetectable concentrations of VTG and significantly lower mean gonadal steroid concentrations (T: 430 pg mL^{-1}; E_2: 8 pg mL^{-1}) than F, EF, and EFAF turtles. The NENF females had mean T concentrations (200 pg mL^{-1}) only slightly higher than, and E_2 concentrations similar to, those reported for Loggerhead nesting females in their fourth nesting event in Australia (Wibbels et al., 1990; Whittier et al., 1997). The VTG and T concentrations in the NENF turtles (VTG: 10 mg mL^{-1}; T: 3 ng mL^{-1}) were also much lower than those reported for Loggerheads nesting in Florida in August (Smelker et al., 2014). Based on these studies, we suggest that the five NENF females nested in Florida in 2014, but finished for the season and were returning to their foraging areas by the time of entrainment in the SLNP. It seems unlikely that these females were part of a nearshore resident population due to the low recapture rate of individual Loggerheads and the rarity of adult Loggerhead captures in the SLNP outside of the nesting season, though benthic-foraging adults are distributed throughout the marine and estuarine waters of Florida (Witherington et al., 2006). However, it is also possible that the NENF females were reproductively quiescent adult females traveling between foraging areas due to their tendency to travel close to shore (Dodd and Byles, 2003).

If the NENF females had been reproductively active earlier in the season, then this would suggest that clearance rates for VTG are relatively high when it comes time for the return migration to the foraging grounds. The end of the nesting season in *Chelonia mydas* marks a metabolic shift from the utilization of lipid-based to protein-based substrates (Hamann et al., 2002a). Accordingly, VTG may be used as a source of energy as females resume active feeding, given the hypophagic behavior they exhibit during the reproductive season (Owens, 1980). Though well-fed, captive juvenile Kemp's Ridley sea turtles exhibited elevated concentration of VTG for two months following large pharmacological E_2

doses (Heck et al., 1997), adult females with endogenous E_2-induced VTG production may exhibit a different pattern, especially because of the high metabolic costs of migration and nesting (Wallace et al., 2005; Hatase and Tsukamoto, 2008). It was reported that sea turtles sampled immediately after returning to the feeding areas following a nesting season still contained mature follicles (Miller and Limpus, 2003). However, nesting *C. mydas* with "depleted" ovaries at the end of the egg-laying season have been reported, which contained fewer than five follicles detected by the ultrasound (Blanco et al., 2012). Licht (1982) described problems with assuming circulating concentrations of a hormone (and presumably, proteins like VTG) based on gross appearance of the ovary. In lizards exposed to captivity stress, "drastic" reductions in VTG concentration occurred prior to the cessation of follicular growth (Morales and Sanchez, 1996), and so it seems that it may be possible to observe a change in VTG concentration prior to complete resorption of ovarian follicles. Additionally, plasma calcium and triglycerides have shown a rapid decrease during ovarian regression in other turtle species (Sarkar et al., 1996). Since VTG was undetectable in females that had completed nesting for the season, we suggest that females utilize circulating VTG and its metabolites resorbed from the ovarian follicles to supplement their diet and fuel their migration back to foraging areas. Thus, VTG may be used not only as a source of energy for the developing embryo, but also by post-nesting females as they reinitiate feeding.

The five NENF females had significantly lower mean gonadal steroid concentration than F, EF, and EFAF turtles. However, the range of concentrations of these steroids overlapped among individuals, and this overlap has also been described in reproductively active Green sea turtles (Allen et al., 2015). Two individuals out of five in the NENF category had a T concentration that overlapped with concentrations seen in reproductively active females, and three NENF individuals out of five had E_2 concentrations that overlapped with concentrations seen in reproductively active females. This overlap may introduce some error into studies that attempt to use gonadal steroid concentrations as an indicator of ovarian activity in Loggerhead females. Inclusion of VTG in these studies could help resolve such confusion since VTG was not detectable in animals that were not reproducing. Consequently, the measurement of VTG in conjunction with the gonadal steroids provides valuable information regarding ovarian activity in adult, female sea turtles.

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