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Plasma Vitellogenin and Testosterone in Diamond-backed Terrapins *(Malaclemys terrapin)* during the Nesting Season in Coastal New Jersey

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Vitellogenesis is the process in which female oviparous vertebrates synthesize the protein vitellogenin to develop egg yolk. In some species, vitellogenin has been used to investigate reproductive status, as a biomarker of clutch size and nesting frequency, and as a biomarker of exposure to endocrine-disruptive chemicals. The Diamond-backed Terrapin (*Malaclemys terrapin*) is an obligate coastal species that in the northern extent of its range nests in the spring and summer months. Diamond-backed Terrapins serve as a key indicator species of coastal ecosystem health; thus, furthering our understanding of the endocrine control of reproduction may inform biologists of the health and status of coastal ecosystems. The objective of this study was to quantify baseline values of vitellogenin and testosterone in Diamond-backed Terrapins during the nesting season, in the northern part of their range. Blood samples were taken from adult female terrapins from two populations in coastal New Jersey from June–August. Enzyme-linked immunosorbent assays (ELISAs) were used to quantify vitellogenin (VTG) and testosterone (T) across the nesting season. VTG concentrations showed peak values in the earliest part of the nesting season, significantly declining through the summer before reaching basal values in August, with T showing a similar trend. This suggests that terrapins in New Jersey follow a similar reproductive cycle to other turtle species from temperate latitudes. Additionally, we found that larger females exhibited higher concentration of T and VTG than smaller females. This suggests that VTG and T are useful biomarkers of reproductive output in these animals. Lastly, we also noted that larger females tended to nest earlier in the nesting season than smaller females. We hypothesize that larger females may compete for resources more effectively and efficiently than smaller females, which may confer larger individuals a fitness advantage.

VITELLOGENESIS is the process in which female oviparous vertebrates form and store nutrients that are required for the development of egg yolk (Ho et al., 1982; Wallace, 1985). After vitellogenin (VTG) is synthesized in the liver, it is transported via the blood stream to the growing oocytes, where it is cleaved into major proteins (lipovitellin and phosvitins) that serve as nutrients to support the developing embryo (Ho et al., 1980; Wallace, 1985; Kuchling, 1999). Vitellogenin synthesis is stimulated by estrogens (typically estradiol-17β) from ovarian follicles, although multiple hormones are thought to play a role in the regulation of this process (Cree et al., 1991; Palmer and Palmer, 1995; Heck et al., 1997). Given this association with estrogens and other sex hormones, VTG can serve as a key indicator of reproductive status and phenology (Ho et al., 1982; Myre et al., 2016) and even of endocrine disruption (Hansen et al., 1998; Tada et al., 2004).

Estrogens are synthesized from androgens through a variety of chemical pathways (Crews et al., 1978; Tsai et al., 1994). Perhaps most commonly, estradiol-17β is synthesized by aromatization of testosterone (T). T itself is a frequently used biomarker of the reproductive cycles of both male and female reptiles (Edwards and Jones, 2001; Taylor et al., 2004; Currylow et al., 2013; Viana et al., 2014) and may help regulate the vitellogenic cycle (Owens, 1980; Ho et al., 1982).

Numerous species of chelonians in both temperate and tropical regions have had their gonadal and hormonal cycles described (e.g., *Chrysemys picta*—Klicka and Mahmoud, 1977; *C. picta*—Ganzhorn and Licht, 1983; *C. picta*—Mitchell, 1985; *Chelodina oblonga* and *Chelodina steindachneri*—Kuchling, 1988; *Caretta caretta*—Wibbels et al., 1990; *Pseudemydura umbrina*—Kuchling and Bradshaw, 1993; *Podocnemis expansa*—Freneau et al., 2017). In general, ovaries develop during summer and fall, and complete maturation occurs before ovulation in late spring/early summer, entering a period of quiescence after the nesting season (Ganzhorn and Licht, 1983; Kuchling and Bradshaw, 1993). However, a unique North American species, the Diamond-backed Terrapin (*Malaclemys terrapin*), has been historically underrepresented in this regard (Holliday et al., 2018), with descriptions of the endocrine cycles in terrapins only being published in recent years (Winters et al., 2016; Donini et al., 2018, 2021). Terrapins are the only turtle in North America exclusively found in brackish water and exhibit a geographic distribution from the Atlantic Shoreline of Cape Cod, Massachusetts to the Texas Gulf coast. Seven taxonomic subspecies are currently represented within this range (Carr, 1952), though newly recognized genetic groupings may alter this status (Hart et al., 2014). Terrapins occur in both temperate and sub-tropical regions, and their reproductive patterns have been described to some extent in some parts of their geographic range.

Most species of turtles in northern latitudes appear to follow temperate or post-nuptial reproductive cycles, where gametes begin production shortly after the nesting season and continue at varying degrees until the following nesting season (Licht, 1982; Kuchling, 1999). In typical post-nuptial patterns, some sex steroids spike at the onset of gonadal recrudescence, followed by a gradual decrease, with multi-clutch species like terrapins undergoing multiple peaks in

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some reproductive hormones (Callard et al., 1978; McPherson et al., 1982; Currylow et al., 2013; Donini et al., 2018). Vitellogenin often follows similar trends, with elevated concentrations during the nesting period, gradually decreasing over the course of the reproductive season, before increasing again in the fall in preparation for the subsequent nesting season (Gapp et al., 1979; Duggan et al., 2001).

The quantification of VTG and T in the context of the ovarian cycle of reproductively active terrapins will allow us, in time, to further describe the reproductive cycle terrapins follow, and may ultimately provide a better understanding of other aspects of their ecology and physiology. For instance, vitellogenesis requires the mobilization of energy reserves and thus, VTG may serve as a biomarker of maternal body condition (Kwan, 1994; Irwin et al., 2001; Hamann et al., 2002).

The northern Diamond-backed Terrapin subspecies *Malaclemys terrapin terrapin* ranges from North Carolina to Massachusetts (Lovich and Hart, 2018). At these northern latitudes, both metabolic and physiologic cycles are regulated by seasonal environmental variations, such as the onset of winter temperatures and other climate conditions (Williard and Harden, 2011; Harden et al., 2015). To endure extreme low temperatures, northern terrapins submerge themselves within the mud of coastal marshes during the winter, entering brumation until temperatures rise, before beginning spring courtship in preparation for the upcoming reproductive season (Yearicks et al., 1981; Brennessel, 2006; Haramis et al., 2011). Northern terrapins typically begin nesting in early June, with nesting continuing through early August (Burger, 1977; Feinberg and Burke, 2003). Clutch size in northern terrapins ranges on the average between 5.3 and 16.1 eggs per clutch, each female generally producing two clutches (Lovich et al., 2018).

The objective of this study was to define seasonal patterns in ovarian activity during the nesting season of Diamond-backed Terrapins by quantifying VTG and T via the examination of reproductively active terrapins in the coastal marshes of New Jersey. Additionally, we assessed the relationship between these reproductive biomarkers and the morphology of terrapins from a life history context. We hypothesized that circulating VTG and T concentrations in terrapins would mirror post-nuptial patterns seen in other multi-clutching chelonian species living in similar climates, with peak concentrations occurring early in the nesting season (i.e., June), gradually decreasing toward the end of the season (e.g., August).

MATERIALS AND METHODS

Study site: Cape May Peninsula.—The Cape May Peninsula area, situated in southern New Jersey, is a location dominated by tourism and surrounded by barrier island beach communities and extensive salt marshes. We conducted our fieldwork near The Wetlands Institute in Stone Harbor, New Jersey. This salt marsh is 4–5 km wide and continuous for approximately 35 km, parallel to the Atlantic Ocean coastline from Cape May, New Jersey to Ocean City, New Jersey (Wood and Herlands, 1997). This habitat is a mixture of sinuous creeks and open shallow sounds, with intertidal marsh vegetation (*Spartina* spp.) dominating (Wood, 1997). On the ocean side of the marsh, there are barrier beach island resort communities, densely populated by residents during the nesting season.

Study site: Hackensack Meadowlands.—The Hackensack Meadowlands, located in northern New Jersey is one of the largest urban estuarine centers found in northeastern United States, containing 34 km^2 of wetlands (Kiviat and MacDonald, 2002, 2004). This area serves as important habitat, as it is home to a diverse community of wildlife. It is home to over 260 species of birds, 22 species of mammals, over 51 species of fishes, 51 species of bees, and 420 species of plants (Kiviat and MacDonald, 2004). The Hackensack Meadowlands are composed of estuarine deep-water habitats permanently submerged by at least 2 m of water at low tide, and shallow habitats located between the estuarine deep water and mudflats. Deep-water habitats are characterized by a lack of vascular vegetation, while shallow water habitats are characterized by a high diversity of salt marsh vegetation, including Saltmarsh Cordgrass (*Spartina alterniflora*), Marsh-fleabane (*Pluchea odorata*), Dwarf Spike-rush (*Eleocharis parvula*), glasswort (*Salicornia* spp.), Spike Grass (*Distichlis spicata*), Switchgrass (*Panicum virgatum*), and Seaside Goldenrod (*Solidago sempervirens*; Kiviat and MacDonald, 2002).

Sample collection.—At the Hackensack Meadowlands site, terrapins were captured using baited Maryland-style crab traps. Each sampling period, 6–12 traps were baited. We used Maryland-style crab traps constructed of vinyl-coated hexagonal mesh wire and measured 61 × 61 × 53 cm with menhaden (Wood, 1997). Traps were submerged for 2–4 hours to eliminate terrapin mortality. At the Cape May Peninsula site, samples were collected from nesting females found on The Wetlands Institute nature trail after the nesting process had been completed. Blood samples were collected from all mature females from early June to early August. Two terrapins from the Hackensack site were recaptured and sampled twice in subsequent months (initially sampled in June, and then additionally in July/August). These samples had significantly different concentrations of VTG and were treated as independent samples; the first sample had VTG at midrange concentration, whereas the second sample had non-detectable VTG. It was not possible to examine the animals internally via X-rays or ultrasound imaging. Blood samples were collected via the subcarapacial sinus using a heparinized 25-gauge needle and 3 ml syringe. Approximately 0.5–1.5 ml of blood were drawn from each female, depending on mass. After collection, samples were immediately placed on ice for up to four hours. After blood was collected, morphological data were recorded (carapace length and width [cm], plastron length [cm], shell height [cm], mass [g], and scute anomalies) with unmarked turtles receiving an internal passive integrated transponder (PIT tag) for mark–recapture purposes. Within four hours after collection, samples were centrifuged at 10,000 rpm for 10 minutes to separate plasma, then stored at –20°C in a Summit medical freezer. At the end of the nesting season, samples were transported from New Jersey to Louisiana frozen on dry ice, before being stored at –80°C prior to vitellogenin quantification.

Vitellogenin ELISA.—We used an in-house vitellogenin ELISA that we developed for emydid turtles, using a primary polyclonal antibody that was derived against Red-eared Slider

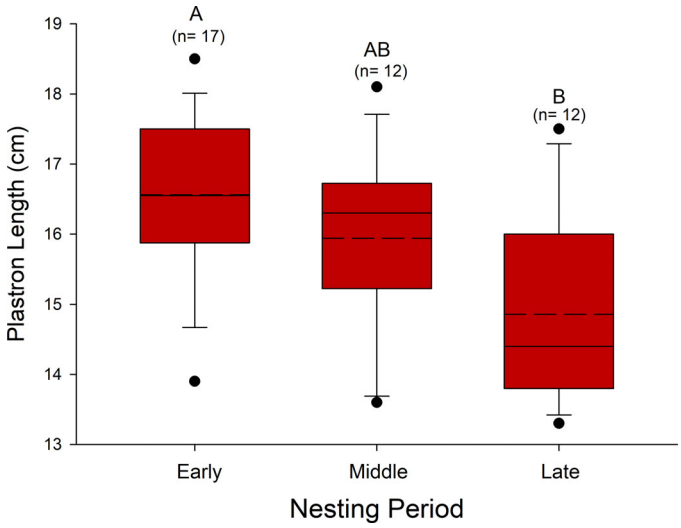

Fig. 1. Mean plastron length (± SEM) of Diamond-backed Terrapins captured during early (June), middle (early July), and late (late July–early August) nesting season. Dashed lines within each box indicate mean of data, while solid lines inside the boxes indicate the median. Varying letters indicate significant differences ($P < 0.05$) between sampling periods. Mean and median overlap in some categories, and both lines may not be visible.

(*Trachemys scripta*) vitellogenin that shows significant cross-reactivity with terrapin VTG (Donini et al., 2018). To run each assay, we pipetted purified VTG standards and terrapin samples in duplicate (100 µl per well) into 96-well flat bottom polystyrene plates (Thermo Fisher Scientific, Inc.). We diluted purified vitellogenin from *T. scripta* (160,000 ng/ml) with phosphate-buffered saline (PBS) in a ten-point two-fold dilution series, starting at 40,000 ng/ml as top dose to build standard curve. We diluted early nesting female samples at 1:30,000, and diluted samples from the middle of the season at 1:20,000 so that binding would be within the linear part of the curve. We added PBS alone to wells as blanks. We incubated plates overnight at 4°C. The following day, we removed solutions and washed plates three times with 150 µl 1X PBS each time. We blocked wells with 100 µl of PBS-blotto (5% g/v nonfat dry milk in PBS). We incubated plates for two hours at room temperature. An hour into incubation, we pre-absorbed the primary antibody (PBS-blotto containing rabbit anti-vitellogenin antiserum and a negative control sample containing no vitellogenin [plasma from male *Lepidochelys olivacea*, at a 1:10 dilution]). We washed plates three times with PBS wash buffer as above and added 100 µl of the antibody at a 1:40,000 dilution into individual wells. We incubated plates for two hours at room temperature with gentle shaking. We washed plates three times with PBS again and added 100 µl of the secondary antibody (PBS-blotto containing goat anti-rabbit, 1:5,000 dilution) to individual wells. To develop the plate, we used TMB Peroxidase EIA Substrate Kit (BioRad Laboratories, Inc.). We incubated plates at room temperature for 10 minutes with gentle shaking. We stopped the reaction after 10 minutes with 100 µl of 1 N H_2SO_4. We then read plates using a Bio-Tek Powerwave HT Microplate Spectrophotometer at 450 nm. Vitellogenin concentrations were calculated using SigmaPlot 13.0 software. We estimated the detection limit as two standard deviations of the mean vitellogenin concentration that was calculated from the mean blank absorbance readings for each plate. We calculated inter-assay and intra-assay coefficient of variation (CV) based on a pool sample repeated three times in each plate.

Testosterone ELISA.—We measured T concentration using a commercial kit (Enzo Life Sciences, Farmingdale, NY) as per the manufacturer's specifications, as described previously (Donini et al., 2018). 50 µl plasma samples were extracted twice with 500 µl of diethyl ether, dried under a stream of nitrogen gas in a 37°C water bath, and reconstituted with 250 µl sample buffer. Each sample was run 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 significant parallelism with the standard curve.

Statistical analyses.—To test the relationship between biometric measurements and biomarkers of reproduction, a two-way analysis of co-variance (ANCOVA) was used to assess interactions between season and site, with plastron length used as a covariate. Variable interactions were not significant ($P > 0.05$); therefore, we conducted a one-way analysis of variance (ANOVA) for each variable to test for differences during each sampling period. We considered $P < 0.05$ significant in our analyses. Additionally, a one-way ANOVA was used to compare the effect of hand capture and trap capture methods on circulating testosterone.

All data were either log transformed or ranked to meet the assumptions of homogenous variance and normal distribution. A single outlier in the late sampling period was removed due to studentized residual value greater than three (Stevens, 1984; Thode, 2002). Linear regression was used to examine relationships between VTG, T, and plastron length.

We performed statistical analyses using SYSTAT (v13.0, Systat Software, Inc., San Jose, CA, USA). All figures were created SigmaPlot (v14.0, Systat Software, Inc.). We report statistics as ± standard error of the mean (SEM).

RESULTS

During the summer of 2013, we collected a total of 41 blood samples from reproductively mature/nesting females. These females were designated as having a midline plastron length greater than 13 cm, based on the smallest reproductive female observed on Little Island, New Jersey (Montevecchi and Burger, 1975).

All ($n = 16$) but one nesting female caught early in the nesting season were from the Cape May Peninsula site, with a single female from the Hackensack Meadowlands. Four nesting females from the middle of the season were from Cape May, while eight nesting females were from the Hackensack Meadowlands. All nesting females from late in the nesting season ($n = 12$) were trapped at Hackensack Meadowlands.

Female size.—Mean midline plastron length of all adult female terrapins was 15.87±0.22 cm (range = 13.3 to 18.5 cm). A significant relationship between plastron length and nesting period occurred, with significantly larger females nesting earlier in the season (Fig. 1; $F_{2,37} = 6.393$, $P = 0.004$).

Plasma vitellogenin and testosterone.—The standard curve for the VTG assay used to analyze terrapin samples ranged from

0.00062 mg/ml to 76.7 mg/ml. We estimated the mean detection limit for this assay at 0.00054 mg/ml ($n = 10$). The overall inter-assay CV was 18.15% and the mean intra-assay CV was 4.42% ($n = 10$). For the T assay, the intra-assay CV was 6.6%.

Nesting female samples were arbitrarily split into three periods to facilitate statistical analysis: early nesting season (June), mid-nesting season (early July), and late nesting season (late July–early August). Circulating plasma VTG concentrations in nesting female terrapins peaked in the early nesting season and decreased over the course of the season (Fig. 2B; $F_{2,37} = 4.546$, $P = 0.00001$). Mean VTG values in females that nested early, mid, and late in the season were 41.67±4.23 mg/ml ($n = 16$), 28.03±2.28 mg/ml ($n = 12$), and 0.001±0.00007 mg/ml ($n = 12$), respectively (Fig. 3).

Plasma T showed peak values early in the nesting season and decreased to the lower limits of the assay in the middle and late nesting season (Fig. 2A; $F_{2,31} = 8.109$, $P = 0.001$). Mean T values in females that nested early, mid, and late in the season were 207.78±57.61 pg/ml ($n = 16$), 14.55±1.34 pg/ml ($n = 8$), and 15.22±1.44 pg/ml ($n = 12$).

There was a significant positive relationship with midline plastron length between both VTG ($r^2 = 0.229$, $P = 0.002$) and T ($r^2 = 0.209$, $P = 0.006$; Fig. 3). No significant differences existed between T concentration and capture method ($F_{1,32} = 1.076$, $P = 0.307$).

DISCUSSION

In this study, both VTG and T followed a similar pattern, with peak values occurring early in the nesting season, before declining through to the end of the season. This reproductive pattern is similar to those observed in temperate chelonian species (Gapp et al., 1979; Duggan et al., 2001; Saka et al., 2011; Currylow et al., 2013), along with those from tropical species (Currylow et al., 2017). Compared to the limited data from other terrapin populations, the trend in T output observed in this study was in agreement with that documented in an unpublished thesis by Lee (2003) in terrapins from South Carolina, along with that observed by Winters et al. (2016), at a similar latitude in Barnegat Bay, New Jersey, with T showing a steady decline from earlier samples across the nesting season. These higher concentrations of T are often associated with large ovarian follicles, and may be associated with some breeding and nesting behaviors (Licht et al., 1979). Winters et al. (2016) observed higher concentrations of T in pre-oviposition terrapins on the nesting beach compared to terrapins that had finished nesting, suggesting T as a possible driver of nesting behavior.

Interestingly, the trend observed in this study is somewhat different from that observed in southwest Florida, where terrapins exhibited elevated T concentration throughout summer sampling (Donini et al., 2018). In studies of terrapins in both Florida (Donini et al., 2018) and Louisiana (Donini et al., 2021), VTG exhibited a similar trend to what we observed in New Jersey. Trends in Louisiana showed comparable results, with peak values occurring in the early nesting season, then decreasing at the conclusion of the season. In Florida, VTG showed similar overall trends but the fluctuations in VTG were limited from May through July, with monthly averages never approaching basal values. Terrapins in southwest Florida likely follow a modified form

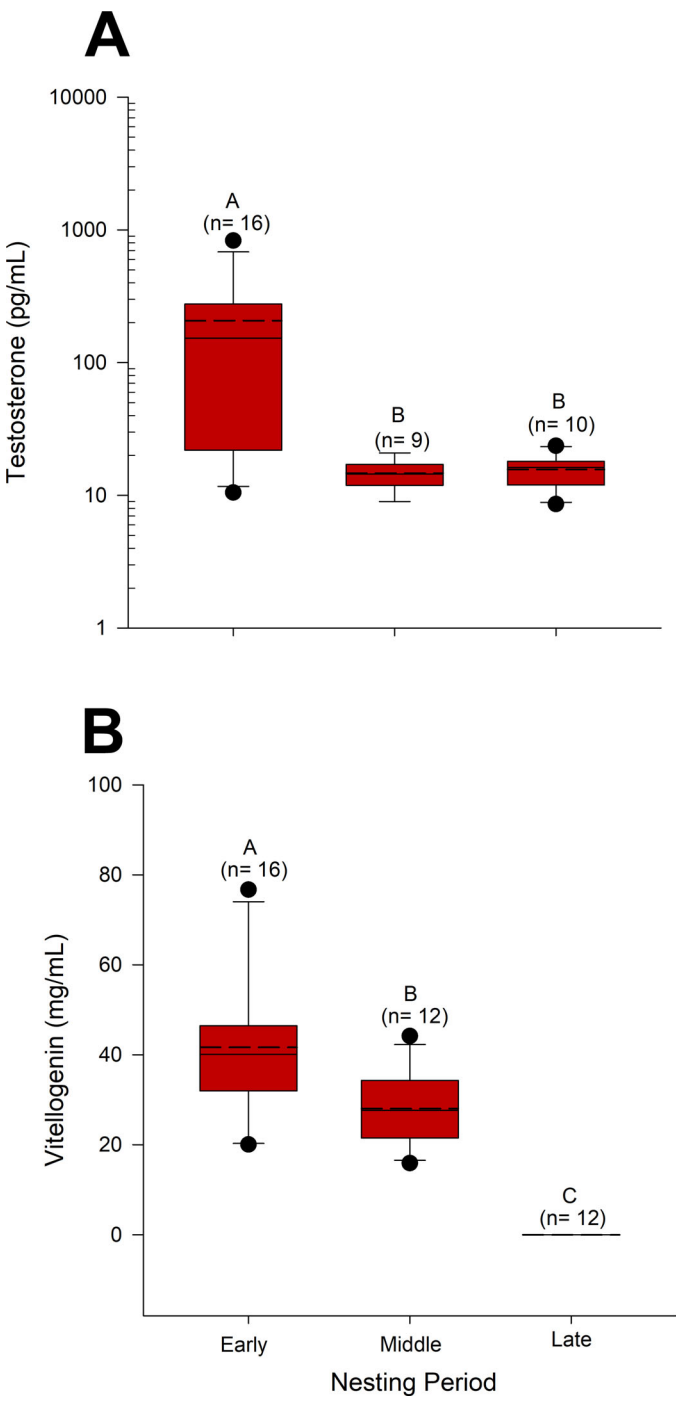

Fig. 2. Mean circulating plasma (A) testosterone concentration (± SEM) and (B) vitellogenin concentration (± SEM) of Diamond-backed Terrapins captured during early (June), middle (early July), and late (late July–early August) nesting season. Dashed lines within each box indicate mean of data, while solid lines inside the boxes indicate the median. Varying letters indicate significant differences ($P < 0.05$) between sampling periods. Mean and median overlap in some categories, and both lines may not be visible.

of the temperate reproductive cycle with extended vitellogenesis and reproductive opportunities due to the absence of harsh winter conditions in the subtropical climate.

Based on our VTG and T data, it is likely that New Jersey terrapins are following closely typical temperate reproductive patterns, where steroidogenesis, and subsequently vitello-

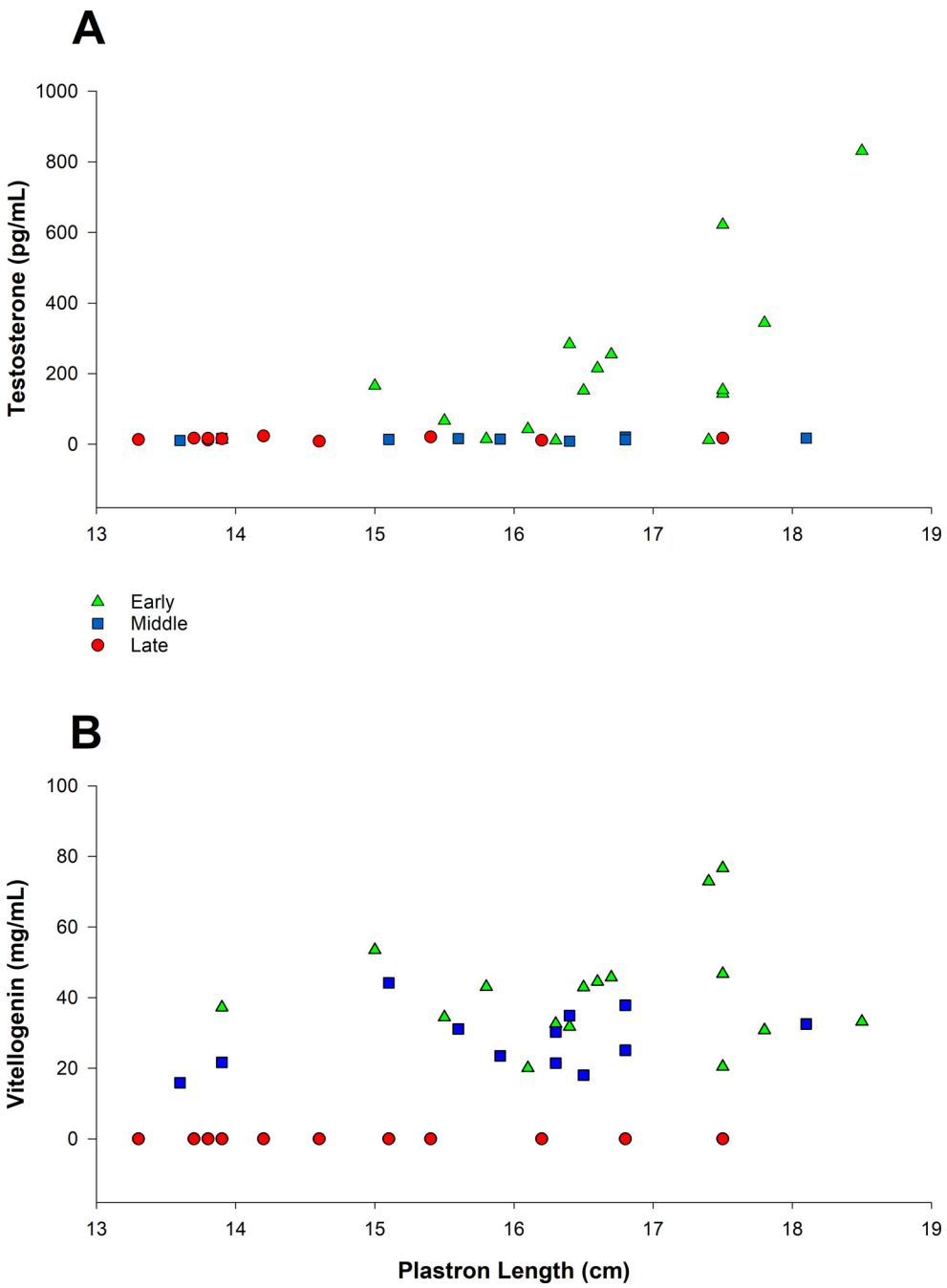

Fig. 3. Plasma testosterone (A) and vitellogenin (B) concentrations in relation to midline plastron length in Diamond-backed Terrapins captured during early (June; green triangles), middle (early July; blue squares), and late (late July–early August; red circles) nesting season at the Cape May Peninsula, New Jersey and the Hackensack Meadowlands, New Jersey.

genesis, decrease as the reproductive season proceeds and the animal enters into a quiescent phase, as indicated by low and basal values of T and VTG observed by the end of the nesting season. This decrease in T is consistent with the regression of the ovaries as the season ensues (Mendonca and Licht, 1986; Wibbels et al., 1990; Rostal et al., 1996). We hypothesized that plasma VTG and T concentrations in terrapins would follow post-nuptial patterns seen in temperate multi-clutch species, with peak concentrations occurring early in the nesting season, gradually decreasing over the course of the season. Our initial hypothesis was supported by our results, as we observed distinctive peaks and progressive declines in T and VTG across the nesting season. Our findings are consistent with previous research in turtles, where in the late spring and early summer VTG concentration declined after ovulation (Duggan et al., 2001; Saka et al., 2011; Currylow et al., 2013; Smelker et al., 2014). Additionally, our findings indicate that VTG is a reliable biomarker of reproductive activity of oviparous vertebrates, as it conforms to a clear profile of reproductive timing.

Interestingly, we observed a significant positive relationship between both VTG and T with plastron length, where larger females exhibited significantly elevated VTG and T concentration compared to smaller females. This supports the hypothesis that VTG and T may serve as biomarkers to describe relationships between size and reproductive output in terrapins, with larger individuals possibly producing larger quantities of T and VTG. Larger females have been documented producing larger clutches of eggs in terrapins (Seigel, 1980; Roosenburg and Dunham, 1997), as well as in other

species of turtles (Frazer and Richardson, 1986; Iverson, 1991; Valenzuela, 2001; Rasmussen and Litzgus, 2010), though some of this is attributed to latitudinal variation in life history strategies (Lovich et al., 2018). Elevated T and VTG concentration could potentially be associated in larger females with clutch size and number of eggs given the prior stated association of these biomarkers with gonadal recrudescence and egg production. Moreover, VTG, in particular, could be associated with foraging quality and quantity, since VTG production may be a function of the accumulation of nutrients, parameters that we did not include in our study. These are aspects of the reproductive biology of terrapins that require additional study across populations to determine physiological influences on life history traits, a data-deficient topic in most studies.

With regard to turtle morphology and reproductive phenology, we observed an interesting relationship between plastron length and nesting period, with a general trend of larger females nesting earlier than smaller animals, with a gradual decrease in plastron size towards the end of the season. Montevecchi and Burger (1975) also investigated the relationship between female plastron size and nest timing, but they did not find any significant relationship between these variables. Life history variations and tradeoffs have been studied at length in a number of turtle species (Congdon and Gibbons, 1985; van Buskirk and Crowder, 1994; Shine and Iverson, 1995). However, to our knowledge, this trend of larger females nesting earlier in the reproductive season has not been observed in any other chelonian species, though it has been documented in both amphibians (Tejedo, 1992) and lizards (Bauwens and Verheyen, 1985), and could have major life history implications for the species. This could be indicative of several key components of life history modifications, including the acquisition and allocation of energy. In many organisms, energy reserves are allocated to survival and growth until maturity is reached, at which point an energy allocation shift towards reproduction occurs (Kozłowski, 1992; Congdon et al., 2003; Harms et al., 2005). Specifically in Diamond-backed Terrapins, females showed slower growth and later reproductive maturity, along with larger estimated asymptotic size in comparison to males in some populations (Harden et al., 2021). Larger females thus may be older and may have acquired skills that allow for more efficient location and consumption of food resources compared to small younger females, allowing large females to allocate energy to reproduction earlier in the season, as feeding and body condition have been documented as key factors in the activation of reproductive cycles and reproductive output in ectotherms (Jørgensen, 1975; Bonnet et al., 2001; Doody et al., 2003; Litzgus et al., 2008).

Further studies should be conducted to investigate the relationship among age, size, and reproductive timing in a larger sample of females in various locations. Overall, this study provides a physiological context to further understand the reproductive cycle of terrapins in its northern range of distribution, providing baseline data for trends in reproductive cycles, as well as generating new hypotheses with life history implications. Further investigations on the relationship of clutch size, VTG, sex steroids, and reproductive output would allow us to obtain an improved understanding of the life history of the only estuarine turtle species of the United States.

DATA ACCESSIBILITY

ACKNOWLEDGMENTS

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