Trends in ovarian development, plasma vitellogenin, and steroid hormones in female *Malaclemys terrapin* (Schoepff, 1793) from coastal Louisiana

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Abstract.—The timing of reproductive cycles in reptiles is often linked to environmental correlates, including temperature and photoperiod. The Diamondback Terrapin (*Malaclemys terrapin*) is a wide-ranging species that occurs across multiple climatic regions over the eastern and Gulf coastal areas of the United States. Therefore, the species may show variation in reproductive cycles according to latitude. To assess the reproductive cycles of *M. terrapin* and to improve our understanding of their range-wide variations, terrapins were sampled in the state of Louisiana during the known nesting seasons from May–August (peak and late nesting period) and outside of the typical nesting season during the month of October (fall pre-wintering period). Terrapins were sampled via ultrasonography to assess the development of ovarian follicles and eggs. Blood samples were collected, and the egg yolk protein vitellogenin and the steroids testosterone and estradiol were measured. Large pre-ovulatory class follicles were present during the peak nesting season, though the animals showed fewer (or lacked) follicles in the later portion of the reproductive season. Pre-wintering samples in the fall showed pre-ovulatory class follicles. Vitellogenin varied significantly across sampling periods, with peak values occurring during the early portions of the nesting season before decreasing in the late nesting season, followed by increases in the fall pre-wintering period. The testosterone concentration did not vary over any of the sampling periods, while estradiol varied significantly across sampling periods, with peak values in the fall pre-wintering period. These results suggest that *M. terrapin* in Louisiana likely follow a post-nuptial reproductive pattern, similar to conspecifics and other emydid turtle species at more northern latitudes.

Keywords. Diamondback Terrapin, vitellogenesis, ovarian cycle, estradiol, testosterone, reproductive cycle

Introduction

Reproductive cycles in ectothermic organisms are largely seasonal and often initiated by environmental cues, including changes in temperature, photoperiod, and rainfall (Noeske and Meier 1977; Horseman et al. 1978; Whitter and Crews 1987). Turtles, like other ectotherms, have reproductive cycles that follow similar cues (Lewis et al. 1979; Kuchling 1982; Mendonça and Licht 1986; Kennett 1999) and typically fall under one of two major categories, depending on whether gametes are produced prior to (prenuptial cycle) or after (postnuptial cycle) the reproductive season (Licht 1982;

Kuchling 1999a). Gonadal steroid hormones are largely responsible for driving major changes in the reproductive cycle, with reproductive hormones serving as key components of gonadal recrudescence, ovulation, and other reproductive activities (Callard et al. 1976, 1978; Huot-Daubremont et al. 2003; Al-Habsi et al. 2005). Non-steroid molecules, such as calcium and the egg yolk protein vitellogenin (Vtg), may also serve as indicators of reproductive functions (Rostal et al. 2001; Saka et al. 2011). Specifically, the production of Vtg may be used as an indicator of reproductive status and reproductive potential in the female reproductive cycle (Ho et al. 1982; Currylow et al. 2013; Myre et al. 2016).

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Though reproductive cycles have been described in many species of reptiles in terms of both gonadal recrudescence and hormonal profiles, gaps in the available information remain for several species, some which are of conservation concern (Graham et al. 2015). Diamondback Terrapins (*Malaclemys terrapin*) are one such species of conservation interest (Roosenberg et al. 2019) and there are descriptions of gonadal and endocrine cycles (Holliday et al. 2018). Terrapins occupy coastal estuarine habitats across the United States from mid-coastal Texas, along the northern and eastern Gulf of Mexico, and up the Atlantic coastline to Massachusetts (Carr 1952; Ernst and Lovich 2009). Given their expansive range, populations at different latitudes may experience varied seasonal and environmental conditions. Throughout much of their distribution, Terrapins are exposed seasonally to low temperatures (Akins et al. 2014), which may serve as a cue for the production of gametes, similar to other chelonian species (Ganzhorn and Licht 1983; Mendonça 1987). Following emergence from winter dormancy, Terrapin nesting is often observed in the spring through summer (Seigel 1980a; Feinberg and Burke 2003; Harden and Willard 2012). Active breeding and courting, however, have been observed in both the early fall and during the spring and summer (Siegal 1980b; Estep 2005).

Along the Northern Gulf of Mexico (NGoM), Terrapins appear to typically nest from late April to August (Mann 1995; Borden and Langford 2008; Coleman et al. 2014; Pearson and Wiebe 2018), and they likely are exposed to variable thermal conditions compared to their northern conspecifics, especially in southern latitudes, where they may experience a shorter period of inactivity in the winter. Recent studies in the NGoM have investigated habitat use and reproductive output (Coleman 2011; Pearson and Wiebe 2018), as well as some potential threats (Drabeck 2014; Coleman et al. 2014) to populations in Louisiana, Mississippi, and Alabama. However, endocrine and gonadal cycles have never been described in wild Terrapins from this region.

In Louisiana, and throughout their range, Terrapins have been impacted both historically and in recent years due to direct human involvement, with some populations experiencing notable declines (Gibbons et al. 2001). These impacts include harvesting for food and the pet trade (Enge 2005; Converse et al. 2017), habitat destruction and fragmentation, and accidental by-catch (Wood and Herlands 1997; Lovich et al. 2018). Therefore, the objective of this study was to investigate the reproductive cycle of Terrapins in Louisiana, by using ultrasound and blood sampling, to document ovarian activity with regard to vitellogenin and sex hormone concentrations. An improved understanding of the reproductive physiology of Terrapins in this region could be valuable for informing stakeholders and conservation agencies in their decision making towards conservation goals. Because most known strategies in temperate chelonians exhibit post-nuptial reproductive cycles, we suspect that Terrapins in Louisiana will exhibit this same pattern, with peaks in hormonal and vitellogenin contents showing elevated concentrations during the nesting period, then declining as the season progresses before increasing again in the pre-wintering period in the fall.

Materials and Methods

Study Sites

Terrapins sampled for this study came from two subpopulations in Louisiana. The first population sampled was at Rockefeller Wildlife Refuge, with sampling occurring in both early and late May 2015 and again in October 2015. A second population was sampled from June–August 2015 in Terrebonne Parish during nesting surveys. Primary habitat in these Louisiana study sites consisted of Smooth Cordgrass (*Spartina alterniflora)* and Saltgrass (*Distichlis spicata*) marshes, as described by Selman and Baccigalopi (2012) and Selman et al. (2014), and pocket shell hash beaches in bay estuaries, as described by Pearson and Wiebe (2018). Terrapins across the state of Louisiana show similar genetic structure throughout the region (Petre et al. 2015), and the populations sampled are at similar latitudes. Thus, data for the two populations were combined during analyses.

Sample Collection

Terrapins were sampled using three methods: (i) manual surveys from airboats for locating mud burrows and mobile Terrapins, (ii) using single lead fyke nets stretched across tidal bayous of saltmarshes (Selman and Baccigalopi 2012, 2014), and (iii) opportunistic hand capture along nesting beaches. Four females which were captured and held by a local fisherman for sale and aquacultural purposes were sampled in August, but it is unknown how long they had been in captivity. The keeper provided no information on light or temperature cycles, but the animals were observed in large Rubbermaid stock tanks in a storage room with artificial lighting. According to the keeper, all animals were readily feeding on invertebrates and fish.

Once captured, females were then bled from the subcarapacial sinus or dorsal coccygeal vein within five minutes of handling (to minimize stress response) using a heparinized 22-gauge needle and 3 ml syringe. A 0.5–1.5 ml sample of blood was collected from each individual, depending on its size. Some Terrapins were bled after a minimum period of 12 h contained in fyke nets, and the parameters were compared between these individuals and those sampled by active methods to assess any stress effects on the hormone values. Whole blood was immediately placed on ice and centrifuged at 3,000 rpm within 3 h of collection. Plasma was kept on dry ice while in the field, before storage at −80 °C.
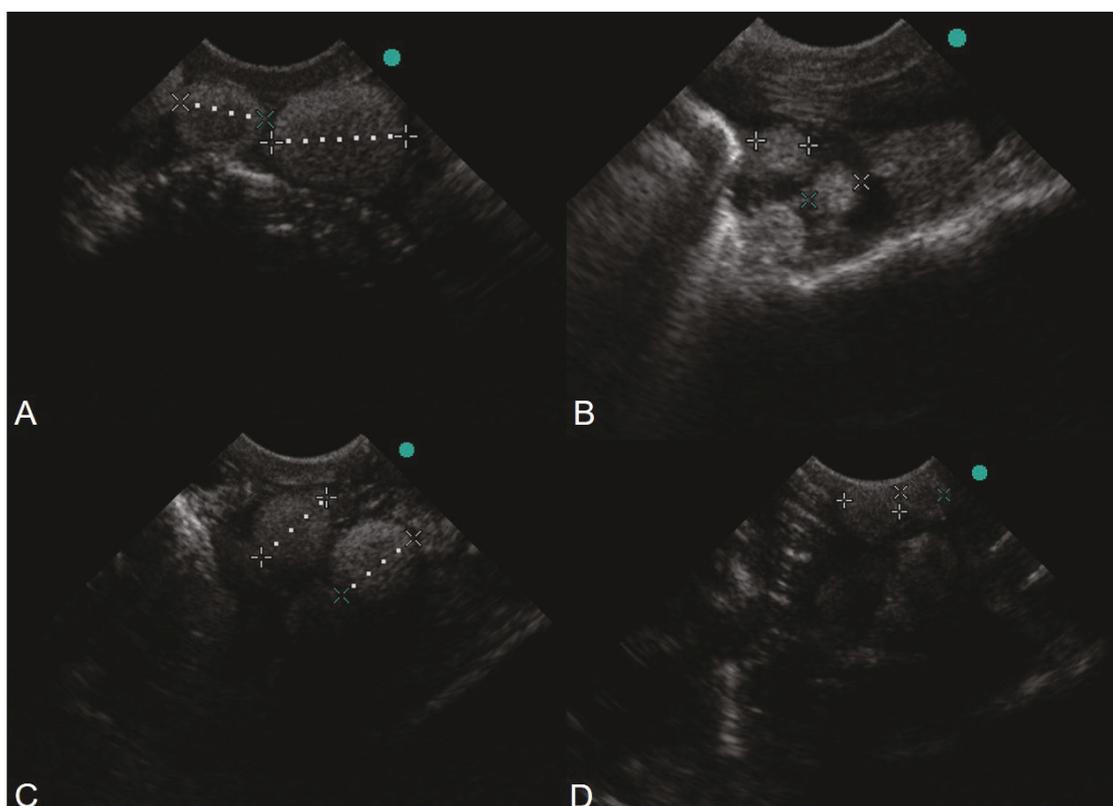
Fig. 1. Examples of various follicular size classes from different sampling periods of Diamondback Terrapins. **(A)** Class II and IV follicles from late May. **(B)** Class I follicles from August samples. **(C–D)**. Class II and III follicles from October samples.

Ultrasound

A portable ultrasound (TITAN, Sonosite Inc., Bothell, Washington, USA) with a 5–8 MHz microconvex transducer was used to examine ovarian development and the presence of follicles/eggs in a subset of captured females. Ovarian follicle diameter was measured to the nearest 0.1 cm using digital software calipers or using the program ImageJ (Schneider et al. 2012). Follicles were assigned to one of four size classes: Class I (< 0.6 cm), Class II (0.6–1.0 cm), Class III (1.1–1.6 cm), or Class IV (> 1.6 cm), adapted from similar classifications by Lahanas (1982) and Mitchell (1985) (Fig. 1). No follicles were detected in four of the female Terrapins (n = 2 in July and n = 2 in August), so these four individuals were excluded from subsequent follicle analysis.

Hormone and Vitellogenin Concentrations

A modified version of the in-house Vtg enzyme-linked immunosorbent assay (ELISA) was used as described by Smelker et al. (2014), using antibodies developed against *Trachemys scripta* Vtg. This antibody exhibits cross reactivity with turtles of the family Emydidae, including *M. terrapin* (Donini et al. 2018). A high-sensitivity commercial ELISA kit (Enzo Life Sciences, Farmingdale, New York, USA) was used for measuring both testosterone (T) and estradiol (E_2). Hormone kits were validated

for this species via parallelism tests of *M. terrapin* plasma. Samples were run in triplicate, at extraction volumes from 50–400 µl depending on season and assay. Hormone extractions were performed via the methods described by Myre et al. (2016) and Smelker et al. (2014) using double ether extractions. Predicted hormone values were corrected for the amount of plasma used for extraction and buffer used for reconstitution prior to analysis. Animals with hormone concentrations below detectable limits (BDL) were assigned a concentration of 50% of the lowest detectable limit for the assays (0.007 ng/ml for E_2 and 0.0013 ng/ml for T), as has been used in other assays and experiments of similar design (Flewelling et al. 2010). Sample sizes varied for analyses of hormone and Vtg concentrations, due to variations in available plasma volume from each sample.

Statistical Analysis

A two way Multivariate-Analysis of Variance (MANOVA) was used to determine whether there were any effects on the results due to variations in sampling location, and additionally for capture type (hand vs. trap capture) to account for potential stress effects on the hormones. Sampling periods were combined based on the period in the known nesting season. Samples from May and a single June sample were categorized as peak nesting period, while July and August samples were

grouped together as late nesting season, and October samples were categorized as pre-wintering. Levene's and Shapiro-Wilk's tests were used to assess normality and homogeneity of variance among sampling periods for the variables (average follicle diameter, Vtg, T, and E_2). Follicle diameter and T data met all parametric assumptions with no transformations required and were analyzed using an analysis of variance (ANOVA). However, Vtg and E_2 data did not meet the assumptions of normality and homogeneous variances, and their skewedness was not within proper parameters to proceed with normal parametric analysis. As a result, non-parametric Kruskal-Wallis analyses with a post-hoc Dwass-Steel-Chritchlow-Fligner test were used to determine variations of Vtg and E_2 across sampling periods. All analyses were performed with SYSTAT 13.0 and all graphical figures were generated using Sigmaplot 14.0.

Results

Validation of sampling sites and methods. All Terrapins sampled were adult females, and were captured during the nesting season (May–August, n = 30) as well as during periods outside of the known nesting season (October, n = 9). There was no significant interaction between season and sampling location ($P > 0.05$), providing additional support for treating both sampling regions as single populations for the analysis, and thus independent ANOVA were used for each variable. Analysis of hand captured and trap captured Terrapins determined there were no significant differences between T and E_2 concentrations with regard to capture type ($F_{1,16} = 3.010$ $P = 0.102$ and $F_{1,32} = 3.179$ $P = 0.084$, respectively).

Ultrasound. A subset of 29 females were sampled via ultrasound. The average follicle diameter did not significantly vary throughout the sampling periods ($F_{2,25} = 2.234$; $P = 0.128$). Mean follicle size during the peak nesting period was 1.23 ± 0.09 cm, and all four size classes were observed in May and June. In the late nesting period samples, individuals either had very small Class I or II follicles, or they lacked follicles completely (mean follicle size: 0.714 ± 0.05 cm). Mean follicle size in the October pre-wintering sample was 1.08 ± 0.11 cm, again with multiple size classes present (Fig. 2). No evidence of atretic follicles was observed in any sampling period. Calcified eggs were visualized in both peak and late nesting periods, but not in the fall pre-wintering periods.

Hormone and vitellogenin concentrations. Thirty-nine individuals were sampled for Vtg, while 21 were sampled for T, and 34 for E_2. The average Vtg intra-assay coefficient of variation (CV) was 4.9% and the inter-assay CV was 14.4%. The average T intra-assay CV was 8.66% and the inter–assay CV was 10.58%. The average intra-
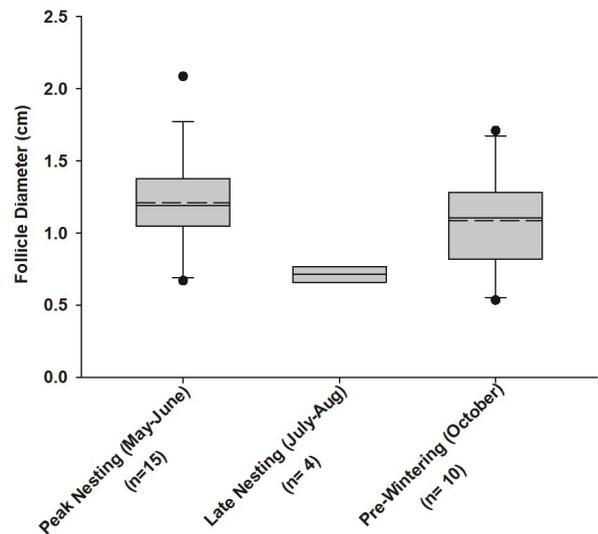

Fig. 2. Ovarian follicle diameter throughout sampling periods of female Diamondback Terrapins captured in Louisiana. Dashed lines within each box indicate the mean of the data, bold lines within each box indicate the median of the data, and the upper and lower edges of the boxes represent the 75% and 25% quartiles. T bars represent the minimum and maximum values. Solid black circles represent outliers. Note n = 4 for late nesting period, but only two samples were used in the analysis as the others lacked any detectable follicles. The mean and median overlap in some categories, so both lines may not be visible.

assay CV for E_2 was 9.74% and the inter-assay CV was 12.3%. Samples from Louisiana were run concurrently with the Florida samples reported in Donini et al. 2018, explaining the similar CV results reported in both papers. Terrapins had detectable concentrations of Vtg during all sample periods, ranging from 0.97– 71.95 mg/ml, and significant differences in Vtg were observed across sampling periods ($\chi^2 = 7.724$; $P = 0.021$; df = 2; Fig. 3A). The highest values were observed during the peak nesting season (28.7 ± 3.06 mg/ml for May and June) with a drop occurring in the late nesting period (July and August, 14.9 ± 7.65 mg/ml). There was no significant difference observed between the late nesting season and pre-wintering samples (October, 17.47 ± 1.04 mg/ml). Likewise, there was no significant difference in T concentrations between sampling periods ($F_{1,19} = 2.541$ $P = 0.127$; Fig. 3B). Mean T values were 119.53 ± 34.04 pg/ml in the peak nesting period, and 47.61 ± 13.90 ng/ml the pre-wintering sampling periods. Samples from July and August had low plasma volumes, which prevented their inclusion in the analysis of T for the late nesting period. There was significant variation in E_2 values across sampling periods ($\chi^2 = 6.567$; $P = 0.037$; df = 2; Fig. 3C), with a decrease from the peak nesting period (4.90 ± 1.67 ng/ml) to the late nesting period (1.06 ± 1.06 ng/ml), followed by an increase in the pre-wintering period (21.48 ± 9.07 ng/ml). Data are summarized in Table 1.
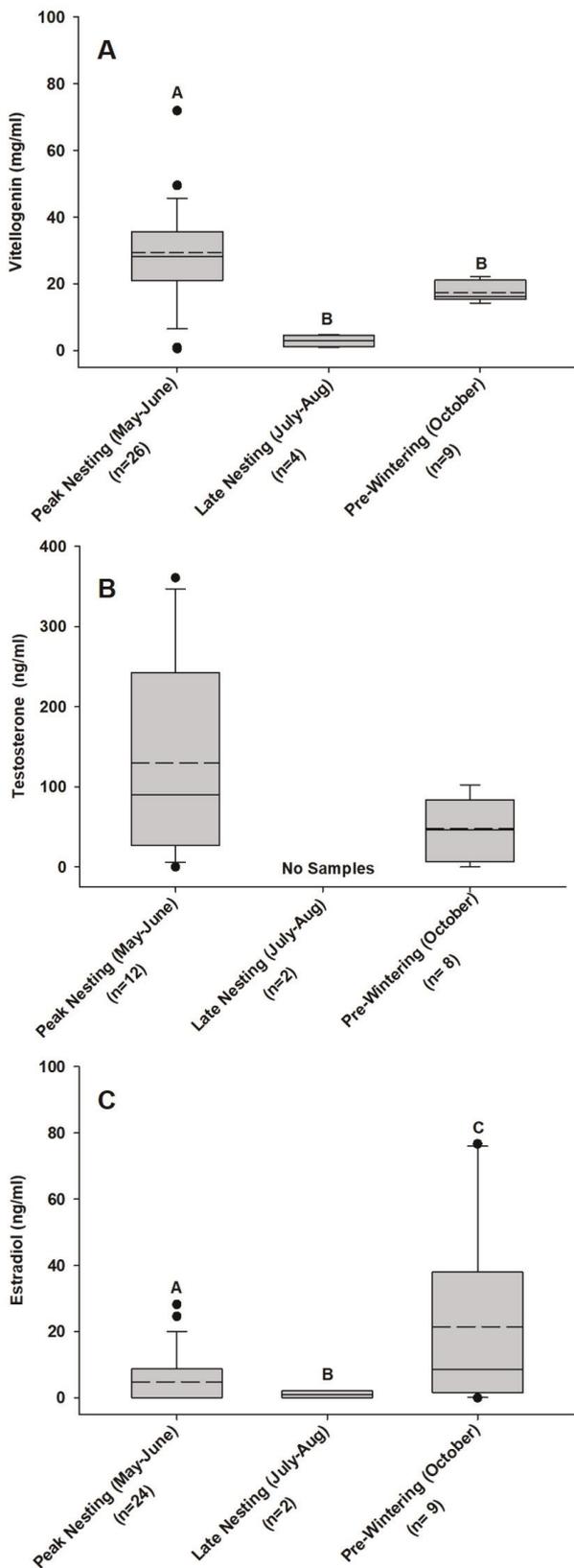
Discussion

Seasonal follicular cycle. All stages of follicles (I–IV) and calcified eggs were present through the majority of the peak nesting period in May and June. Despite being gravid, females either lacked follicles or showed only Class I or II follicles during the late nesting season. Fall pre-wintering samples showed Class I–III follicles. However, no significant differences between average follicle size existed among sampling periods. This general trend in follicular changes throughout the season is indicative of the post-nuptial cycle observed in most temperate turtle species, where large follicles are more common in the early nesting periods, before being ovulated and/or reabsorbed as the nesting season concludes, with gonadal recrudescence occurring in the months following final oviposition (Lewis et al. 1979). In the late nesting season samples, no females showed large preovulatory class follicles, suggesting that their reproductive output for the season had likely ended. The small Class I follicles in two of the August samples likely represent the beginning of the development of the following year's clutch, similar to patterns in other temperate species (Gibbons 1968; Ernst 1971; Robinson and Murphy 1978; Ganzhorn and Licht 1983).

Seasonal vitellogenin concentrations. The Vtg concentrations were found to be higher in the peak nesting season (early May–July) and began to drop as the nesting season concluded. Though not statistically significant, the increasing pre-winter Vtg concentrations is suggestive of fall follicular proliferation. This apparent "rise" in the fall, along with the previously presented follicular increase in size, suggested that follicular recrudescence likely begins in the transition period between summer and fall, with the Vtg concentration increasing at this time, allowing for follicular growth. In Louisiana, we expected the Vtg trends to resemble those of northern turtle populations, as Vtg should be highest during the peak of the reproductive season, before decreasing as the season concludes. Indeed, this trend is similar to those reported in *Chrysemys picta* (Duggan et al. 2001; Gapp et al. 1979) and in Terrapins in New Jersey (Wolfe 2014) as the nesting season reached its end in late summer.

An important factor to consider is that the four individuals sampled in August of the late nesting period were collected by commercial fishermen and retained for a potentially extended period of time. It is not known how long these individuals were in captivity, but they were confirmed to have been captured during the same sampling year. Nonetheless, these individuals may have

Fig. 3. Concentrations of vitellogenin (**A**), testosterone (**B**), and estradiol (**C**) throughout sampling periods of female Diamondback Terrapins captured in Louisiana. Dashed lines within each box indicate the mean of the data, bold lines within each box indicate the median of the data, the upper and lower edges of the boxes represent the 75% and 25% quartiles, and bars represent the minimum and maximum values. Solid black circles represent outliers, and different letters indicate significant differences ($P<0.05$) between sampling periods. The mean and median overlap in some categories, so both lines may not be visible.

skewed our data for this period. Because these individuals either lacked or had only small previtellogenic or Class I or II follicles upon ultrasound examination, this coincided with the decrease in circulating Vtg. However, reabsorption of developing follicles has been documented in wild pleurodire species, such as *Psuedemydura umbrina,* when brought into captivity (Kuchling and Bradshaw 1993). The stressors that the collected terrapins experienced could have potentially altered their natural cycles. At least two of the individuals in the fisherman's possession were captured on nesting beaches and showed retained eggs. The retention of eggs and dystocia (egg binding) have both been linked to environmental and captivity stressors (Buhlman et al. 1995; Kuchling 1999b; Innis and Boyer 2002), which may have influenced the natural vitellogenic cycles of these animals. However, as noted by Kuchling (1999b), some species of turtles are less prone to have altered reproductive output under captive stress. Close relatives of the Terrapins (e.g., *Chrysemys picta* and *Trachemys scripta*) along with dissimilar species, like *Sternotherus odoratus,* are resilient species and capable of producing eggs in captivity, even after removal from the wild (Mendonça and Licht 1986; Bowden et al. 2001). The lower concentrations of Vtg observed here in August and the fall corresponded with the findings of Wolfe (2014), who also found that Terrapins exhibited a quiescent period in the late summer/fall as nesting was completed. Therefore, while potentially impaired due to captivity, we believe the values from captive individuals likely mimic the natural cycle, as they did not deviate from the values reported in other studies.

Seasonal sex hormone concentrations. Testosterone was found in relatively high concentrations in both the early nesting season and pre-wintering sampling periods, corresponding to large steroidogenic follicles that were detected during the same time. However, there was no significant difference in the T concentrations between periods. Winters et al. (2016) noticed a distinct decline in the T concentration of female Terrapins as the nesting season progressed in New Jersey, something our data was unable to capture given the lack of plasma available for assays and sample size as a whole. Lee (2003) showed peak T values occurring in April, before decreasing in May and June, in a population of Terrapins in South Carolina. Lee (2003) also documented basal values of T from July to August, before the T concentration increased steadily from September to October. Other temperate species, such as *Graptemys flavimaculata,* also exhibited increased T in the fall and spring, before it decreased in late summer (Shelby and Mendonça 2001). Terrapins from Louisiana may exhibit a similar pattern, but our missing sampling points during the late nesting season prevented confirmation of this pattern.

Estradiol was found in low concentrations during the early nesting period, and even lower (near the detectable limit) during the late nesting season (two samples in July). These concentrations are drastically lower than those reported for similar periods in Florida Terrapins (Donini et al. 2018). The concentrations then increased to peak values in the fall pre-wintering period. This hormonal trend is further suggestive of a postnuptial cycle initiated in fall, with females beginning follicle development for the following nesting season, similar to the observations of Lee (2003) in South Carolina. It is also possible that the low concentrations of E_2 observed could be explained by the difference in sampling methods, as some of the Terrapins in this study were captured via fyke nets, making it difficult to know capture time prior to sampling. Thus, the low concentration of reproductive steroids observed may be explained by stress artifacts, as glucocorticoids may affect the concentrations of other hormones in reptiles (Mahmoud et al. 1989; Elsey et al. 1991). However, when these trap-captured samples were compared to those that were hand captured, no significant difference existed between the two groups. Further, the high concentrations of T in similar sampling periods indicated that stress may not have altered the estradiol concentrations observed in trap-captured animals. Additionally, some vertebrates release sex hormones in brief periods and small concentrations. For instance, only a single large peak of E_2 was observed in *G. flavimaculata* at a similar latitude in the middle of their known nesting season (Shelby et al. 2001). E_2 specifically is only needed in small doses to initiate vitellogenesis via exogenous injections (Ho et al. 1981), so it is possible that the concentrations detected were sufficient to stimulate vitellogenesis for a subsequent clutch. This possibility is further supported by peak concentrations of Vtg during the same time period, indicating that vitellogenesis was at its peak. Additionally, it is possible that T was still in the process of aromatization at the time of sampling in these individuals. Aromatization of T into E_2 is a known pathway in the endocrine cycles of turtles (Tsai et al. 1994; Crews et al. 1996), and the peak estradiol concentration may have been missed. This differs from the observations in Donini et al. (2018) for Terrapins from south Florida, in which E_2 and T concentrations remained elevated simultaneously during the nesting season, coinciding with confirmed multiple clutch productions. Despite following the same overall method of post-nuptial reproduction, this may indicate some differences in reproductive timing in the Terrapins between these two latitudes, though this is only speculative given the gaps in sampling data from earlier spring. The low concentrations of E_2 in two of the Terrapins sampled in the late nesting period samples from July coincided with a lack of follicles, supporting the idea that these animals had finished producing clutches for the season and were entering a quiescent phase.

It is legal to harvest Terrapins in Louisiana during a limited season from 15 April–16 June, but only if the Terrapins have a carapace length of 6 in (15.2 cm) or

greater (Louisiana Fishing Regulations 2020). However, this harvest window overlaps with the major reproductive periods described herein, so it puts reproductively active females at the greatest risk of collection given the timing and harvest size requirements. Consequently, it may be prudent to revisit this law given the findings of this paper and other researchers concerning Terrapin reproduction along the NGoM to ensure the future survival of this species in the state.

Conclusions

This study is one of very few which quantifies the endocrinological and ovarian dynamics of the reproductive cycle in *M. terrapin*, and it provides novel data for the species in the NGoM. Overall, the data presented here suggest a brief late summer quiescent phase before ovarian recrudescence begins in the fall, with vitellogenesis occurring into the spring, resulting in the production of at least one clutch of eggs with the potential for more. Information gaps still remain in our knowledge of the endocrine and ovarian cycles of *M. terrapin* in this data set, and for the species as a whole. However, based on the results from this study, we now have baseline data that suggest a temperate pattern for the post-nuptial cycle in female Terrapins in the NGoM. It is possible that conspecifics at higher latitudes follow similar trends, but additional sampling in these regions, as well as during more sampling periods, is necessary to complete a full description of the reproductive cycle of this species. Though limited, the data presented here do provide some clarity on the reproductive season and activity of *M. terrapin* in Louisiana, and may prove useful in the potential amendment of collection laws regarding the size and timing of collection in Louisiana.

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