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Comparisons of Summer and Winter Patterns in Ovarian Development, Plasma Vitellogenin, and Sex Steroids in Female Diamondback Terrapins (*Malaclemys terrapin*) in Southern Florida

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ABSTRACT. – The reproductive cycles of turtles are linked to environmental factors, such as photoperiod and temperature. Currently, the reproductive physiology of diamondback terrapins (*Malaclemys terrapin*) is poorly understood, especially in Gulf of Mexico. The reproductive cycles of terrapins are thought to follow typical seasonal patterns. However, latitudinal variations in temperature regimens lead to longer-lasting warm periods, which can facilitate extended reproductive periods in some turtle species. This suggests that terrapins may show a similar change in the southern parts of their range. To elucidate aspects of the terrapin reproductive cycle, we sampled during the known reproductive season of a southern population of terrapins (May–July), as well as during the winter in late December and early January. We used enzyme-linked immunosorbent assays to quantify concentrations of the plasma sex hormones estradiol and testosterone, and the egg yolk protein precursor vitellogenin. Additionally, we used radiography and ultrasonography to monitor the ovarian status and egg development in females. Follicles showed no significant difference in average diameter across sampling periods with preovulatory class follicles existing in both summer and winter. Eggs were only detected from May to July, with radiographic data showing second clutches in 4 individuals. Testosterone and estradiol showed elevated concentrations throughout the nesting season, coinciding with multiple clutches of eggs, before both showed a significant decrease in winter. Vitellogenin showed peak concentrations in June with other months showing lower but detectable concentrations. Our results suggest that in southwestern Florida, terrapins may have extended reproductive potential and continuous vitellogenic cycles given the presence of preovulatory follicles and high quantities of vitellogenin found in summer and winter. However, true continuous reproduction was not detected in this study.

KEY WORDS. – vitellogenin; terrapin; estradiol; *Malaclemys*; ovarian cycle; testosterone

The diamondback terrapin (*Malaclemys terrapin*) is the only obligate estuarine chelonian species in the United States ranging from the Texas Gulf of Mexico coast up the eastern seaboard to Cape Cod (Carr 1952; Gibbons et al. 2001). Terrapins use many forms of coastal habitat and may serve as keystone species in coastal ecosystems (Levesque 2000; Cole and Hesler 2001). Terrapins have a history of exploitation by the food trade (Schaffer et al. 2008), with more recent threats such as habitat fragmentation, incidental drowning as bycatch, and collection for the pet trade negatively influencing populations (Bishop 1983; Wood and Herlands 1997; Cheung and Dudgeon 2006; Coleman et al. 2014; Crawford et al. 2014). These pressures and declines in populations have led to terrapins being listed as imperiled or vulnerable in several states (Hackney 2010), while also leading to additional population assessments in both government-based and indepen-

dent studies (Mitro 2003; Butler et al. 2006; Hart and McIvor 2008; Boykin 2011; Selman et al. 2014). Many facets of terrapin life history have been studied; however, our understanding of their reproductive endocrinology, particularly with respect to the Gulf of Mexico populations, is lacking. Further understanding the reproductive cycles of this species throughout its range could lead to better understanding of reproductive seasons, and in turn allow for additional and more appropriate conservation practices to be employed.

Currently, our knowledge of diamondback terrapin reproductive cycles largely comes from populations in more northern latitudes along the eastern seaboard. In these populations, terrapins show a peak nesting period ranging from April to July, before slowing down in August and becoming inactive in the late fall or winter (Seigel 1980; Lazell and Auger 1981; Roosenburg 1994; Wood

and Herlands 1997). Terrapin reproductive cycles are likely linked to temperature and photoperiods, as is the case in other species (Ganzhorn and Licht 1983; Mendonca 1987). However, some species with ranges extending into southern latitudes with mild winter temperatures show extended reproductive potential compared to northern conspecifics (Iverson 1977; McPherson and Marion 1981; Kuchling 1999b).

Many species also show variation of clutch size and egg size across latitudes, including *M. terrapin* (Tinkle 1961; Greaves and Litzgus 2009; Allman et al. 2012). It is possible that terrapin endocrine cycles might show similar variation across latitudinal gradients, with the potential for continuous reproductive cycles in more southern regions with less intense winters (Licht 1984).

Further, most studies of *M. terrapin* have rarely investigated ovarian progression and hormonal production and have almost solely relied on nesting females to assess reproductive activity with the exceptions of thesis studies by Lee (2003) and Wolf (2014), which used hormones, ultrasound data, or proteins to determine reproductive status. Nesting females offer important information into the reproductive activity of these animals but may not tell the full story of reproductive cycles, as differences in hormonal concentrations due to the stress and rigor of nesting may obscure the actual status of reproductive cycles (Winters et al. 2016).

Reproductive cycles in most nonavian reptile species can be described as a derivative of 1 of 2 major categories: prenuptial cycles, in which gametes are produced rapidly just prior to reproductive activities (e.g., mating, nesting), or postnuptial cycles, in which gametes begin developing shortly after the reproductive seasons have ceased (Gorman et al. 1981; Licht et al. 1982; van Wyk 1995). Often, cycles can be diagnosed via the proliferation of ovarian follicles in the case of females, and the growth of testes and production of sperm in males (Gibbons 1968; Georges 1983; Iverson and Moler 1997; Meylan et al. 2002). Steroid hormones such as testosterone (T) and estradiol (E_2) can also serve as key indicators of reproductive cycles, often showing increases that correspond to seasonal reproductive activity and gonadal development (Amey and Whittier 2000; Edwards and Jones 2001; Hamann et al. 2002; Huot-Daubremont et al. 2003). The egg yolk protein precursor vitellogenin (Vtg) is regulated by E_2 , and is deposited into developing ovarian follicles to serve as nourishment for developing embryos once eggs are laid (Cree et al. 1991; Kuchling 1999a). Like steroid hormones, Vtg can be used to monitor the reproductive status of females in oviparous species (Ho et al. 1982; Heck et al. 1990).

The purpose of this study was to elucidate the reproductive cycles of *M. terrapin* in south Florida, using the gonadal steroids T and E_2 , along with Vtg and radiographs and ultrasound imaging of the ovaries. We hypothesized that terrapins in southern Florida would exhibit the ability to reproduce in an extended fashion,

similar to other species with ranges extending across diverse latitudes (Iverson 1977; McPherson and Marion 1981; Moll and Moll 1990; Kuchling 1999b).

METHODS

Study Site. — We sampled terrapins in a single mangrove estuary, predominantly composed of red mangrove (*Rhizophora mangle*) and black mangrove (*Avicennia germinans*), with intersecting tidal creeks that emptied into the Pine Island Sound of Lee County, Florida. Terrapins were sampled in collaboration with an ongoing mark-recapture study with the Sanibel Captiva Conservation Foundation.

Sample Collection. — During the spring and summer of 2014, we sampled for terrapins across 8 d in May (~ 60 survey hours), 7 d in June (~ 50 survey hours), 5 d in July (~ 40 survey hours), and 3 d (~ 25 survey hours) in December and January of 2014–2015. Gravid females were previously documented in this population, between late April and late July (C. Lechowicz, unpubl. data, 2013). Terrapins were sampled exclusively through opportunistic hand capture, taking advantage of inflow and outflow of water from tidal creeks as has been documented in other populations (Tucker et al. 1995). Upon capture terrapins were sexed by external dimorphic characters, such as tail length and head width (Gibbons and Lovich 1990).

Upon first handling, between 0.5 and 2.0 ml of blood was collected from the subcarapacial sinus via a syringe with a heparinized 25-gauge needle. Blood was drawn within 5–10 min of capture. This time frame reduces the potential for stress-induced artefacts as documented in other species (Mahmoud et al. 1989; Owens 1997). Whole blood was put into 1.5-ml Eppendorf tubes and stored on ice immediately after collection. Samples were centrifuged within 3 hrs of collection to separate plasma, which was then stored on dry ice before transport to a -80°C freezer. Animals were measured, tagged with passive integrated transponders, and marked on the external marginal scutes via the Cagle (1939) method before release.

Ultrasound/Radiographic Imaging. — From May to July 2014, female terrapins were transported to the Clinic for the Rehabilitation of Wildlife, Sanibel, Florida, for ultrasound and radiographic examination in order to determine ovarian status and clutch size. During the December–January sampling a portable ultrasound (TI-TAN, Sonosite Inc, Bothell, WA) was used to inspect ovarian development in the field. Egg and ovarian follicle diameters were measured to the nearest 0.1 cm using built-in caliper tools in Sonosite software or post hoc using ImageJ (Schneider et al. 2012) owing to a limit on the number of measurements in the active Sonosite software. Follicles were assigned to 1 of 4 size classes (C) as described by Lahanas (1982) and Mitchell (1985) (C1, < 0.6 cm; C2, 0.6–1.1 cm; C3, 1.1–1.6 cm; C4, > 1.6 cm). Both ultrasounds used convex-type probes (3.5–6.8

Table 1. List of recaptured terrapins by ID and capture month; x represents no sample.

Terrapin ID	First month of capture	Second month of capture	Third month of capture
3	May	July	x
22	May	July	December/January
27	June	July	x
33	June	July	x
48	May	June	x
58	May	July	x
71	May	June	x
85	May	July	x
97	June	July	x

MHz), with the portable Sonosite machine using a slightly smaller microconvex probe.

Hormone and Vtg Assays. — We used commercial enzyme-linked immunosorbent assay (ELISA) kits (Enzo Life Sciences, Farmingdale, NY) for measuring both E_2 and T. High-sensitivity kits (products ADI-901-176 and ADI-900-174) were used to quantify the hormones. Hormone kits were validated for this species via parallelism tests of *M. terrapin* plasma to the standard curve of the assay. Hormone extractions were performed using 150–400 μ l of plasma using the methods described by Myre et al. (2016) and Smelker et al. (2014). Predicted hormone concentrations were corrected for the amount of buffer used for reconstitution prior to analysis. Animals with concentrations of hormones below detectable limits were assigned a concentration of 0 pg/ml.

For Vtg measurement, we used an in-house ELISA similar to that described by Smelker et al. (2014), using *Trachemys scripta* anti-Vtg antibodies, which have shown excellent cross-reactivity among turtles of the Emydidae family, including *M. terrapin* (Wolfe 2014). Briefly, diluted purified Vtg standards and samples using phosphate-buffered saline (PBS) were set up at dilutions ranging from pure plasma to 1:7500. We placed these standards and samples on 96-well microplates and incubated microplates at 4°C overnight. The following day, plates were washed with PBS–Tween 20 solution (100 μ l of Tween 20 per 100 ml of PBS), blocked with PBS Blotto (5 g nonfat dry milk per 100 ml PBS), and incubated at room temperature on a gentle shaker (600 rpm) for 2 hrs. A primary Vtg antibody developed from *T. scripta* was then presorbed at room temperature for 1 hr in 1:1000 PBS Blotto with 12 μ l male terrapin plasma determined to have undetectable concentration of Vtg. This antibody solution was then diluted at 250 μ l:10,000 μ l prior to plating. The plate was washed before being filled with the primary antibody and incubated for 2 hrs at room temperature on a gentle shaker. A secondary antibody, goat anti-rabbit IgG coupled to horseradish peroxidase (Bio-Rad Laboratories Inc, Hercules, CA), was diluted 5 μ l:10,000 μ l and added to the plate after washing, and again allowed to incubate for 2 hrs at room temperature. Following the wash of the secondary

antibody, 100 μ l of 3,3',5,5'-170 tetramethylbenzidine peroxidase ELISA substrate kit substrate (Bio-Rad) was added to each well and allowed to incubate for 10 min. The subsequent reaction was stopped using 100 μ l of 1 N H_2SO_4 per well. Following the addition of the stop solution, the plate was read using a Bio-Rad 550 microplate reader at 450 nm. Concentrations were predicted via the standard curve analysis (SigmaPlot 13.0) and corrected by the corresponding dilution factor before analysis.

Statistical Analysis. — A 1-way analysis of variance was used to investigate the relationship between sampling period, follicle size, hormones, and Vtg. We performed a Levene's test to check for homogeneity of variance ($p > 0.05$). Normality of data was tested using a 1-sample Kolmogorov-Smirnov test with Lilliefors p -value ($p > 0.05$). In order to meet the assumption of a normal distribution and equal variances, data for T, E_2 , and Vtg, were rank, log, or square-root transformed if $p < 0.05$. A Tukey's post hoc test or contrasts study was used to discern significant differences between sampling periods ($p < 0.05$). Analyses were performed in SigmaPlot 13.0 and SYSTAT 13.0. All figures display raw untransformed data. Statistics are reported as $\bar{x} \pm SE$.

RESULTS

Capture Effort. — A total of 29 capture events took place of 18 individual terrapins: May ($n = 11$), June ($n = 7$), July ($n = 8$), and ($n = 3$) in late December–January. Nine of these individuals were recaptured in different sampling periods (Table 1), likely because of a small population size with a greater than 60% recapture probability (C. Lechowicz, unpubl. data, 2013). Recaptures that occurred in separate months were treated as independent samples, while 3 individuals that were recaptured within the same sampling month were removed from analysis to avoid pseudoreplication.

Ultrasound/Radiographic Imaging. — Ultrasound data revealed multiple size classes of follicles (C1–C4) across months (Fig. 1). The average follicle diameter did not vary significantly throughout sampling periods, with animals exhibiting C3- and C4-sized preovulatory follicles in each period ($F_{3,16} = 3.238$; $p = 0.815$). Seventy-eight percent of the terrapins ($n = 18$) examined via ultrasound had C3 or C4 follicles. No follicular atresia or ovarian quiescence was detected in any sampling period. Radiography revealed 13 ($n = 18$) gravid females (shelled eggs) from May to July with an average clutch size of 6.15 ± 0.30 eggs. Second clutches of eggs were detected in 4 individuals (Fig. 2), with some of these gravid females showing large C3 and C4 follicles that could yield potential third (or more) clutches. No shelled eggs were detected during winter sampling.

Hormone and Vtg Assays. — T was detectable in all analyzed samples ($n = 25$) with a range of 8.3–412.2 pg/ml. E_2 was detectable in all but 2 samples ($n = 26$) ranging

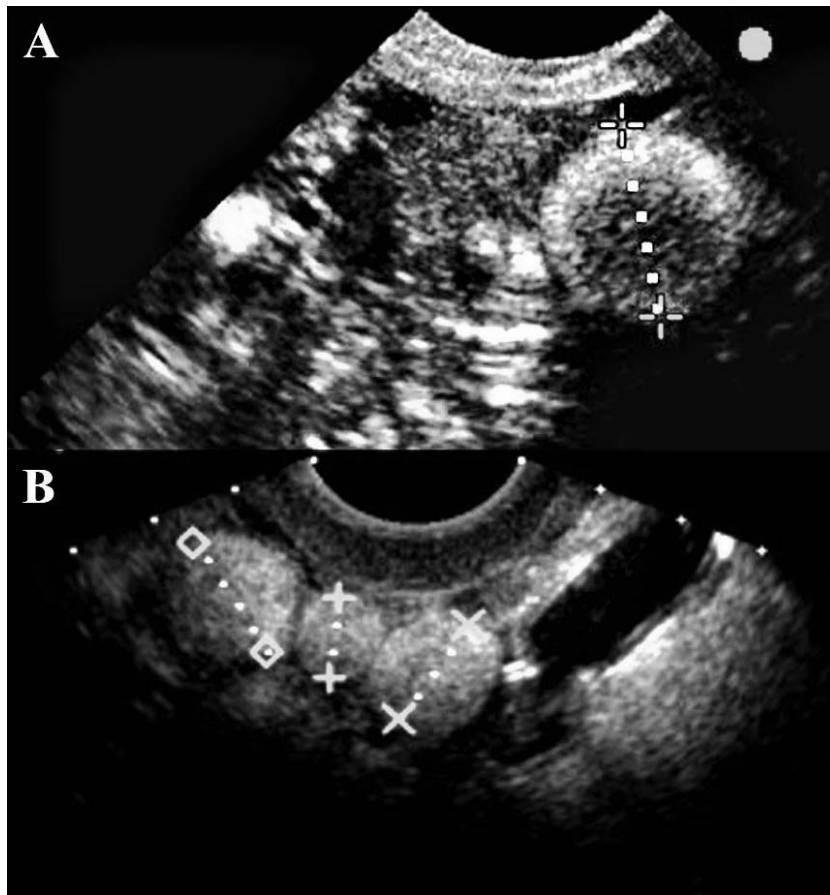


Figure 1. (A) C3 class 1.52-cm follicle as indicated by calipers from the female terrapin (ID 22) in December 2015. (B) C2 and C3 class follicles from same terrapin in June 2014. Follicle size as indicated by calipers from left to right: 1.41 cm (squares), 0.9 cm (plus signs), and 1.59 cm (× symbols).

from 0 to 318.7 pg/ml. Terrapins showed no significant differences in T across the summer sampling period but did show a significant drop during the winter sampling period ($F_{1,21} = 4.611$; $p = 0.044$) (Fig. 3). E_2 showed no significant differences across summer sampling months until a significant decline in concentration was observed in December–January ($F_{1,22} = 5.042$; $p = 0.035$) (Fig. 3). The average T intra-assay coefficient of variation (CV) was 8.66% and the interassay CV was 10.58%. The average intra-assay CV for E_2 was 9.74% and the interassay CV was 12.3%.

All females analyzed ($n = 26$) had detectable concentrations of Vtg ranging from 14.28 to 51.74 mg/ml. Vtg showed significant variation between months sampled ($F_{3,22} = 7.626$; $p = 0.001$). Vtg concentrations showed a significant increase from May to June followed by a significant decrease in July with no significant differences from May or July detected in December–January samples (Fig. 3). The average Vtg intra-assay CV was 4.9% and the interassay CV was 14.4%.

DISCUSSION

Patterns in Ovarian Development and Egg Production. — The presence of large (C3 and C4) preovulatory

follicles and the lack of atretic follicles in any sampling period suggest that ovulation, fertilization, and oviposition could be possible even in the winter months, as has been documented in other closely related species in the Emydidae family (Iverson 1977; Vogt 1990). However, the small sample sizes, particularly during winter months, limit the depth of the conclusions that can be made.

The only other study to our knowledge to examine ovarian development in this species was an unpublished thesis by Lee (2003), in which follicular quiescence was observed in late summer, with recrudescence beginning during the fall in a South Carolina population. In this same study, follicles of preovulatory size (< 1.0 cm) were first documented in the winter, showing little change until ovulation in the spring. This suggests a postnuptial cycle that arrests during winter months. Our Florida data, although limited in scope by comparison, showed no instances of quiescence or atresia in our later samples, and large follicles (> 1.0 cm, designated as C3 and C4 classes) were observed through all sampling periods, consistent with Lee's (2003) observations.

Hormonal Patterns. — T showed no significant difference across summer sampling periods but did show a significant decrease in the December–January sampling period. T has been observed to decrease as the nesting

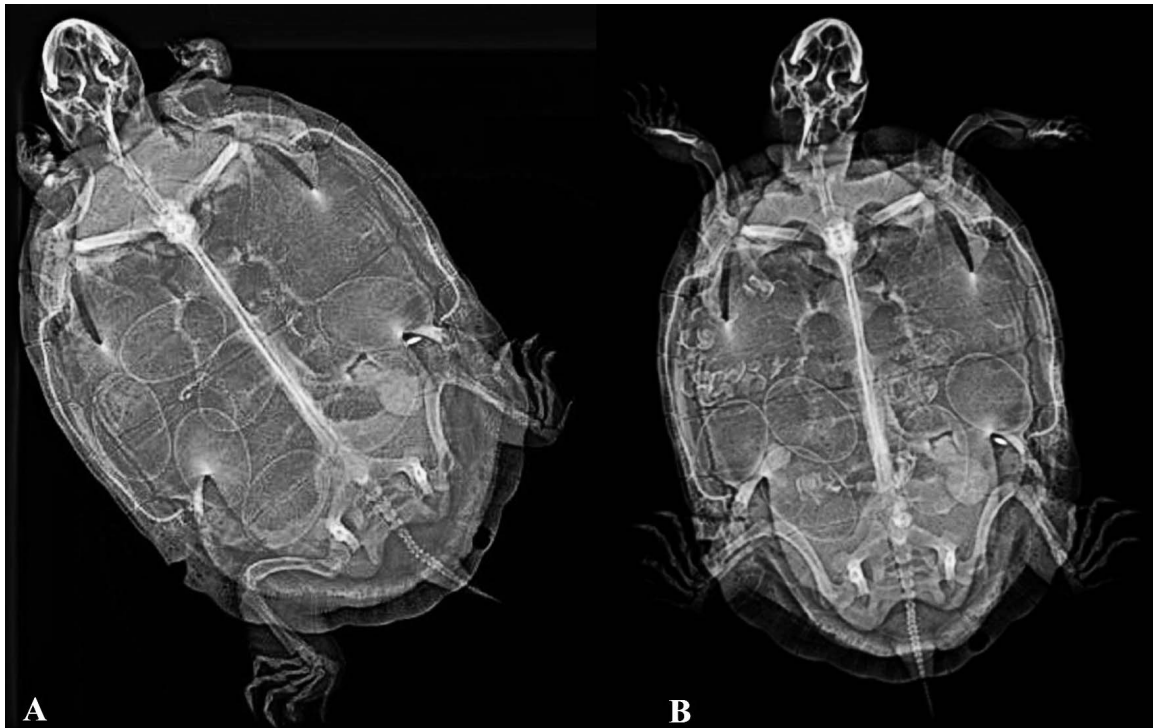


Figure 2. (A) Female terrapin (ID 71) showing a clutch of 6 eggs on 28 May 2014. (B) The same female on 27 June 2014 with a clutch of 5 eggs, signifying a second clutch.

period progresses in northern populations of *M. terrapin* (Winters et al. 2016) and in other turtle species (Licht et al. 1985; Currylow et al. 2013; Myre et al. 2016); however, we did not see the same trend in this population during the confirmed nesting months. This may suggest an extended summer period of reproduction, with a decrease potentially not beginning until later in August or the following fall.

Similarly, E₂ showed sustained concentrations throughout the nesting season in Florida before declining

in December–January. These sustained concentrations during the nesting season correspond to trends seen in other multiclutching species of turtles (McPherson et al. 1982, with elevations in E₂ during the nesting season corresponding to the maintenance of ovarian follicles for the production of additional clutches of eggs. This is further supported by the existence of multiple clutches of eggs in the same females in the May–June sampling period.

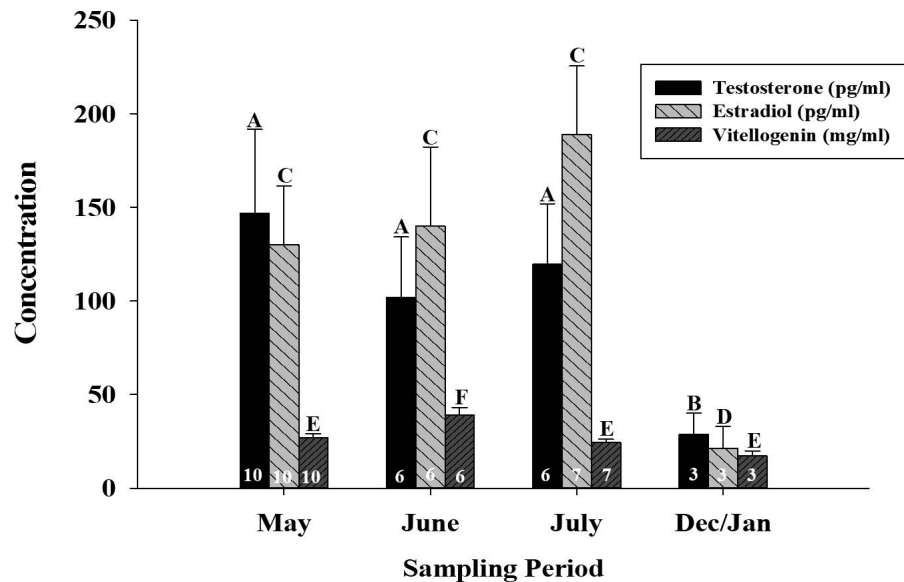


Figure 3. Mean testosterone, estradiol, and vitellogenin concentrations in terrapins across sampling months. Letters indicate significant differences. Sample size indicated by numbers within bars.

The winter decline in both T and E₂ was somewhat unexpected as the animals captured were actively foraging and swimming and presented large presumably steroidogenic preovulatory follicles, suggesting the environmental conditions were acceptable for reproductive activity. However, it is possible that even in a warmer winter scenario, these terrapins may have a hormonal cycle conserved from their more northern relatives, where an arrest in steroidogenesis might occur until the beginning of the nesting period. It is also possible that elevated concentrations of E₂ are not required to sustain follicular proliferation outside of the nesting season, given its potency in chelonians. In a study involving exogenous injections of E₂ in *Lepidochelys kempii*, a small dose has been shown to increase Vtg concentrations for months after the termination of injection (Heck et al. 1997).

Vtg Patterns. — Terrapins were expected to show elevated concentration of Vtg throughout the year as part of a continuous reproductive cycle. However, a significant increase in Vtg was observed in June with a significant decline shown in July and December–January. This is similar to the trend seen by Wolfe (2014) in *M. terrapin* in the northern distribution of their range. In that study, Vtg showed significant decreases from June to July, followed by a further significant drop in early August to below detectable limits (0 mg/ml). Limited ultrasound data were gathered in the Wolfe (2014) study; however, it is highly likely that gonadal quiescence had begun in later summer samples given the large decrease in circulating Vtg. Similar patterns have been documented at the end of nesting cycles in *Chrysemys picta* (Duggan et al. 2001) and *Chinemys reevesii* (Saka et al. 2011). However, in our study no significant differences were observed between May, July, and December–January, with all 3 of these periods showing concentrations well within detectable limits. The small winter sample size may play a role in the lack of significance between some of these other months; however, it should be noted that the range of concentrations in winter samples was 14.3–22.4 mg/ml, values well above basal concentrations and within the range of Vtg concentrations detected during the summer months.

There were no signs of ovarian quiescence or follicular atresia observed in any July-sampled turtles with several individuals exhibiting C4 size class follicles. One female (ID 22) was sampled in both July and December and displayed C3-sized follicles in both instances with Vtg concentrations of 19.3 and 15.1 mg/ml, respectively. This, and the lack of follicular atresia observed in any female turtle, is consistent with the idea that the vitellogenic cycle in the Florida population may be continuous and is extending past typically known periods. This has been documented in some species such as the spotted turtle (*Clemmys guttata*). *Clemmys guttata* females begin developing follicles a year in advance and undergo a continuous vitellogenic cycle with perhaps only a brief dormancy period during winter, while not

ovulating until the spring nesting season (Ernst and Zug 1994).

Conclusions

Here we report a portion of the seasonal reproductive patterns *M. terrapin* exhibit in southwest Florida. Our data suggest that the reproductive cycle of terrapins in southern Florida may be modified in the form of extended reproduction with animals having the potential to produce additional clutches of eggs into and past August given the lack of atretic follicles observed, as well as the high concentrations of Vtg relative to other studies that were observed. This is consistent with the idea of a continuous vitellogenic cycle in these animals; however, without access to data from these turtles in the late summer or fall we cannot be certain.

Although incomplete and with sample-size limitations in some sampling periods, the data collected for this study, provide baseline trends and values for Vtg, gonadal steroids, and ovarian observations for future comparative studies in other populations. The limits of this study highlight the need for continued research in this region and across the range of *M. terrapin*, in order to further our knowledge on the reproductive endocrinology of this species. Endocrinology has been used in variety of ways to both inform and apply conservation tactics to chelonian taxa. Currylow et al. (2017) used both reproductive and stress hormone production as an indicator of potential reproductive success in the critically endangered species, *Astrochelys yniphora*, to help facilitate more efficient captive management of assurance colonies. Fluctuations of hormone concentrations may also serve as indicators of environmental stressors such as contaminants, climate change, and habitat degradation (Crews et al. 1995; Shelby and Mendonca 2001; Cash and Holberton 2005), with baseline data serving as critical information for comparisons in the future. Thus, further understanding the reproductive output and variations in the reproductive cycle of *Malaclemys* could aid in defining conservation objectives and policies, facilitating a wider breadth of protections for the species.

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