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Year-round plasma steroid hormone profiles and the reproductive ecology of gopher tortoises (*Gopherus polyphemus*) at the southernmost edge of their range

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ABSTRACT

Populations of wide ranging ectotherms often exhibit variation in traits that are influenced by local environmental conditions. Although the gopher tortoise, *Gopherus polyphemus*, is well studied in pine flatwoods habitats across their range, little attention has been given to coastal populations existing in the southern extreme portion of the range. We examined the reproductive physiology of a coastal dune population in southwest Florida to determine if reproductive cycles vary across populations. Here we present the first year-round sex hormone profiles for a wild population of gopher tortoises. Male testosterone concentrations varied across the year ($F_{11,54} = 2.52$, $P = 0.015$) with elevated values from September to December and minimal levels from April to July, with the exception of a secondary peak during the month of June. Female testosterone and estradiol concentrations varied across the sampling period (T: $F_{11,66} = 8.54$, $P < 0.001$, E: $F_{11,66} = 4.57$, $P < 0.001$) with highest values from August to February, and lowest levels from May to July. Female progesterone concentrations varied over the year ($F_{11,64} = 3.29$, $P = 0.002$) and increased in late fall with a peak in March. These data suggest this population has an extended breeding season from fall through spring with mating likely occurring from September through March, and nesting in winter through spring. This pattern is similar to reproductive patterns described for tropical and sub-tropical chelonians but differs from that of gopher tortoise populations in northern portions of the range where hibernation may last for five months and a single clutch of eggs are deposited in late spring.

1. Introduction

An organism's reproductive schedule has a considerable influence on individual fitness. Life history theory, therefore, predicts that species will breed when the likelihood of success is maximized. Many species exhibit a plastic reproductive schedule where reproduction occurs during environmental conditions that ensure successful mating and favor survivorship for the offspring (Anderson et al., 2010; Lyon et al., 2008; Reed et al., 2009; Verhulst et al., 2008; Walde et al., 2007). Flexibility in reproduction benefits species living in habitats that vary unpredictably (Caswell, 1983; Kaplan and Cooper, 1984), wide-ranging species whose populations are exposed to different environmental patterns (Caswell, 1983; Norris, 1993; Vogt, 1990), and organisms exposed to the impacts of climate change (Urban et al., 2014; Wright et al., 2012). This paper aims to explore the reproductive schedule of gopher tortoises (*Gopherus polyphemus*) living in the southern-most edge of their range where they do not brumate since our current knowledge of this species' reproductive ecology is entirely based on populations above the freeze line where individuals brumate in the winter (Butler and Hull, 1996; Landers et al., 1980; Ott et al., 2000).

Reproduction in temperate and sub-tropical reptiles is cyclic and associated with temperature, photoperiod, and moisture serving as the primary zeitgebers used by the hypothalamus to coordinate the release of gonadotropins from the pituitary gland, and eventually the sex hormones from the gonads (Ganzhorn and Licht, 1983; Mendonça, 1987). Testosterone concentration in the blood of male reptiles is associated with gonadal recrudescence, with highest concentrations during courtship and spermatogenesis (Licht et al., 1985; Mahmoud et al., 1985; Mendonça and Licht, 1986). Estradiol concentration in the blood of female reptiles is linked to ovarian development, with highest concentrations observed during vitellogenesis (Kuchling, 1999; McPherson et al., 1982; Radder et al., 2001). Progesterone plays a role

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in the final maturation of the follicles, ovulation, and stasis of the myometrium to allow for shelling of the eggs (Callard et al., 1978). This hormone increases in females just after ovulation with additional spikes for each subsequent clutch of eggs (Ho et al., 1981).

Many reptiles exhibit prenuptial reproductive cycles where male sex hormones and spermatogenesis are temporally synchronized with ovulation in females, but most turtles have postnuptial cycles where male reproductive activity does not coincide with ovulation in females (Crews, 1984; Kuchling, 1999; Licht, 1984). In most temperate turtles, estradiol increases in late summer and through early fall as vitellogenesis progresses and egg follicles enlarge (Congdon and Tinkle, 1982; McPherson and Marion, 1981). Estradiol is also elevated in the spring as egg follicles mature just prior to reaching peak concentrations during ovulation. Estradiol concentrations decline and progesterone spikes at the time of ovulation in spring or early summer (Currylow et al., 2013; Licht, 1982; Rostal et al., 1997). Testosterone concentrations increase in male turtles during late summer and fall during spermatogenesis, and again in the spring during mating (Currylow et al., 2013). Spermatozoa are stored in the epididymis through the fall and winter, until mating in the spring (Rostal et al., 1994). In some species, mating occurs in the fall and the sperm are stored in the oviduct until eggs are fertilized just before nesting in the spring (Gist and Jones, 1989; Palmer et al., 1998; Rostal et al., 1994).

Several species of turtles exhibit geographic variation in reproductive characters that reflect differences in environmental conditions experienced throughout their range. The diamondback terrapin, *Malaclemys terrapin*, begins nesting earlier in southern populations and exhibits differences in reproductive output across latitudes (Allman et al., 2012). The Florida cooter, *Pseudemys floridana*, and striped mud turtle, *Kinosternon baurii*, have wide ranges in North America and exhibit extended reproductive seasons in southern populations (Aresco, 2004; Ewert and Wilson, 1996; Iverson, 1977). It is important to know the seasonal hormone concentrations across populations of wide-ranging turtle species to fully understand the mechanisms regulating reproductive cycles and reproductive output.

The gopher tortoise is a well-studied occupant of pine flatwood, coastal scrub, and coastal dune habitats of the Atlantic Coastal Plain in the southeast United States. Populations throughout much of their range are exposed to seasonally cool temperatures that result in a hibernation or dormant period where individuals remain in their underground burrow for up to five months (Diemer, 1992; Douglass and Layne, 1978; McRae et al., 1981). In north Florida, hatchling tortoises are dormant from late September through late March but will periodically emerge to bask during relatively warmer days in the winter (Butler et al., 1995; McRae et al., 1981). These populations exhibit the typical post-nuptial reproductive schedule of temperate turtles where mating begins in early spring after post-winter emergence, and nesting occurring from May through June (Butler and Hull, 1996; Epperson and Heise, 2003; Iverson, 1980; Smith, 1995). On the other hand, mating behavior has also been observed from March through October for populations in Georgia (Johnson et al., 2007; Landers et al., 1980), suggesting sperm from late summer mating events is stored by the female until the following year's nesting season (Moon et al., 2006).

Gopher tortoise populations in the southern portion of their range are exposed to a sub-tropical climate of hot, wet summers and warm, dry winters that rarely reach freezing temperatures (Winsberg, 2003). Year-round foraging opportunities allow individuals in south Florida to experience rapid growth and mature at earlier ages (9–11 years) than individuals in more northern populations (19–21 years) (Douglass and Layne, 1978; Landers et al., 1982; Mushinsky et al., 1994). Since environmental temperature is a primary driver of gonadal cycles, one would expect the reproductive cycles in these southern tortoises to vary from the known cycles of northern populations. A better understanding of their reproductive ecology is needed across their range to highlight regional differences and better inform management practices for this protected species that has experienced significant population declines throughout their range (Auffenberg and Franz, 1982).

In this paper we describe the seasonal reproductive hormone blood profiles for a population in southwest Florida existing at the southern extreme terminus of the species range. We report the concentration of testosterone, 17β-estradiol, and progesterone for adult tortoises for 12 consecutive months and correlate these levels with observations of reproductive behavior and existing knowledge of cycles in more northern populations. To our knowledge, this is the first paper to report year-round hormone profiles for wild gopher tortoises, or any other turtle species in North America.

2. Methods

2.1. Study area

We captured wild, free-ranging gopher tortoises (*Gopherus polyphemus*) at Delnor-Wiggins Pass State Park located in Collier County, Florida. The park is adjacent to the Gulf of Mexico and contains 4.5 ha of beach dune, 5.5 ha of maritime hammock, and 36.4 ha of mangrove tidal swamp that buffers the surrounding estuary. A population of gopher tortoises utilize the dune and hammock habitats that extend 1.7 km linearly (north–south) and only 50 m in width (east–west). Thirty years (1980–2010) of climate data indicates the average daily high temperature in summer is 28.3 °C and the average daily low temperature in winter is 11.8 °C (Norwegian Meteorological Institute). The park is located within the zone 10a of the USDA plant hardiness zone map, indicating the average annual minimum winter temperature ranges from −1 °C to 1.1 °C (United States Department of Agriculture).

2.2. Sampling

We conducted line transect sampling each month to hand capture approximately five male (n = 3–6) and five female (n = 4–7) gopher tortoises through haphazard visual occurrences while the animals were away from burrows. The sex was determined by examination of secondary sexual characters such as length of gular projection and presence of a concave plastron (Mushinsky et al., 1994). All individuals used for this study had a carapace length greater than 23.0 cm since male individuals of this size have fully developed secondary sex characteristics and both sexes are known to be reproductively mature (Diemer and Moore, 1994; Landers et al., 1982; Mushinsky et al., 1994). We captured all animals within a five-day period and used the same time period each month to ensure equal and consistent temporal intervals between each sampling period.

Immediately upon capture, 2–3 ml of blood was collected from the subcarapacial venous sinus within four minutes of capture using a heparinized 3 ml syringe and a 1.5 in., 22-gauge needle (Dyer and Cervasio, 2008; Hernández-Divers et al., 2002). Turtles were released without collection in cases that blood could not be collected within the four-minute time frame necessary to prevent stress artifacts in the blood samples (Fazio et al., 2014; Moore et al., 1991). Blood was immediately transferred to sterilized microcentrifuge tubes and placed on ice. We collected standard morphometric measurements (carapace length (CL), carapace width (CW), plastron length (PL), mass), GPS location, and individual's unique ID (from ongoing mark-recapture study) before releasing the animal at the site of capture. All procedures followed Florida Fish and Wildlife Commission permit requirements (Permit LSSC-12-00011B), Florida Department of Environmental Protection permit conditions (Permit 09301514), and FGCU's Institutional Animal Care and Use Committee protocol #1314-04.

Blood samples were centrifuged within 30 min from collection using a field centrifuge at 3300 rpm for 10 min. The plasma was pipetted to microcentrifuge tubes, wrapped with parafilm, and placed on dry ice until placed in laboratory storage at −80 °C.

2.3. Hormone Analysis

Progesterone, testosterone, and estradiol concentrations were quantified by radioimmunoassay (RIA) (Bowden et al., 2002; King and Bowden, 2013). Plasma samples were prepared by combining 100 ml of plasma and 300 µl of distilled H_2O. Tracer consisting of 2000 cpm of tritiated progesterone, testosterone, and estradiol (Perkin Elmer, Boston, MA), was added to each sample to allow for the calculation of recovery values, and samples were vortexed and placed at 4 °C overnight incubation to allow for equilibration. Steroids were then extracted using 4 ml of diethyl ether, dried under nitrogen, and resuspended in 10% ethyl acetate in isooctane prior to fractionation using column chromatography. Each steroid fraction was then dried and resuspended in 550 µl phosphate buffer and stored at 4°C overnight. To quantify steroid concentrations, samples were run in duplicate (200 µl each) through a competitive-binding RIA using antibodies specific to each steroid of interest (P = 20R-PR053w, Fitzgerald, Acton, MA; T = 20R-TR018w, Fitzgerald, Acton, MA; E_2 = 7010–2650, Biogenesis, Brentwood, NH), while 100 µl of each sample was used to calculate the percent recovery. Concentrations were calculated based on standard curve the ranged from 3.91 to 1000 pg for progesterone and 1.95 to 500 pg for testosterone and 17β-estradiol. The results from sample duplicates were averaged and then adjusted for individual sample recoveries. Samples were randomized across three different assays. Recovery values averaged 59.6% for progesterone, 62.6% for testosterone, and 57.2% for 17β-estradiol. Intra-assay variation, calculated as the coefficient of variation for the standards, was 7.8%, 3.2%, and 13.9% for progesterone, 5.6%, 3.2%, and 6.0% for testosterone, and 13.1%, 8.3%, and 21.4% for 17β-estradiol, respectively. Inter-assay variation was 14.7% for progesterone, 5.7% for testosterone, and 24.6% for 17β-estradiol.

2.4. Data analysis

We analyzed data using NCSS-12 Statistical Software (NCSS, LLC, Kaysville, Utah, 2018). The Shapiro-Wilk W test was used for each variable to ensure normal distribution. As needed, data were log transformed to meet the assumptions of parametric analyses. Levene's test was used to confirm homogenous variance ($p > 0.05$). Data were screened for extreme outliers using central tendency, dispersion, and stem-and-leaf plots. Two observations were removed because they had Studentized residuals of 4.3 and 15.3, both above the recommended absolute value of 3 (Thode, 2002). In comparison, the average value of the studentized residuals for all other observations was −0.145 (−1.38 to 0.96). Normal probability plots were examined to ensure removed outliers did not impact the data structure. An Analysis of Co-variance (ANCOVA) was initially used to account for body size (covariate) and repeated samples (individual ID) but both variables were not significant ($p > 0.05$). We therefore conducted an Analysis of Variance (ANOVA) for each hormone to test for differences among the means during each sample period (fixed effect). A Tukey-Kramer (TK) post hoc analysis was used for pairwise comparisons of all significant results to identify differences between months.

3. Results

We collected 119 blood samples from adult tortoises between July 2016 and June 2017, with an average of five males (3–6) and five females (4–7) sampled per month. Blood was collected only once from 13 tortoises, and an average of 4.0 times from 28 tortoises throughout the collection period. Male tortoises had a mean CL of 28.7 cm (24–32 cm) and mass of 4.4 kg (2.2–5.8 kg); female tortoises had a mean CL of 30.4 cm (25–34.3 cm) and mass of 5.4 kg (2.9–7.2 kg). The variation in hormone concentrations could not be attributed to body size ($p > 0.05$) or individual ID ($p > 0.05$), indicating these variables did not influence the data structure. While sampling, we observed male courtship behavior (head bobbing) each month of the year and mating behavior (mounting) each month from October through March.

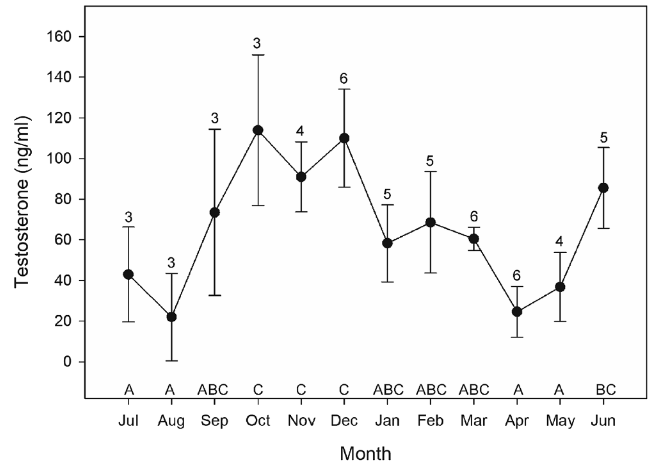

Fig. 1. Mean monthly plasma testosterone concentrations from 54 wild male *Gopherus polyphemus* samples collected from July 2016 through June 2017. Vertical bars represent ± 1SE. Letters indicate significant differences ($p < 0.05$) as indicated by Tukey-Kramer *post hoc* comparisons. Numbers above data points indicate sample size.

Male testosterone varied across sampling periods ($F_{11,54} = 2.52$, $P = 0.015$) with concentrations increasing in September to peak from October through December (mean 105.1 ng/ml ± 13.9) (Fig. 1). Testosterone concentration began to decrease in January to reach minimum concentrations from April through July (mean 46.7 ng/ml ± 9.8), apart from an increase during the month of June.

Female testosterone varied across sampling periods ($F_{11,66} = 8.54$, $P < 0.001$) with concentrations increasing in September to peak from October through March (mean 11.5 ng/ml ± 1.6) (Fig. 2). Testosterone started declining in March and returned to minimum concentrations from May through August (mean 1.5 ng/ml ± 0.3). Female estradiol changed across sampling periods ($F_{11,66} = 4.57$, $P < 0.001$) with relatively higher concentrations from August through February (peak in November) (mean 12.9 ng/ml ± 1.1), before declining to minimal values from May through July (mean 2.9 ng/ml ± 0.4)

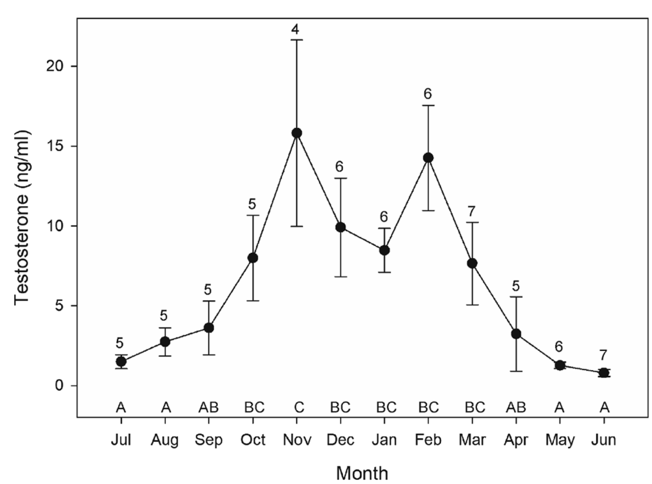

Fig. 2. Mean monthly plasma testosterone concentrations from 65 wild female *Gopherus polyphemus* samples collected from July 2016 through June 2017. Vertical bars represent ± 1SE. Letters indicate significant differences ($p < 0.05$) as indicated by Tukey-Kramer *post hoc* comparisons. Numbers above data points indicate sample size.

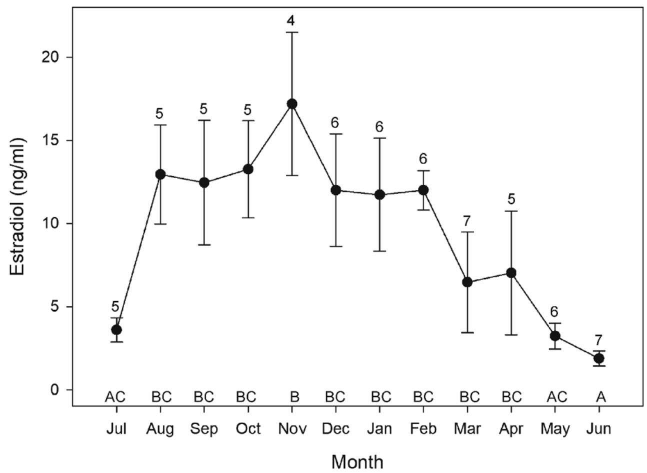

Fig. 3. Mean monthly plasma estradiol concentrations from 65 wild female *Gopherus polyphemus* samples collected from July 2016 through June 2017. Vertical bars represent ± 1SE. Letters indicate significant differences ($p < 0.05$) as indicated by Tukey-Kramer *post hoc* comparisons. Numbers above data points indicate sample size.

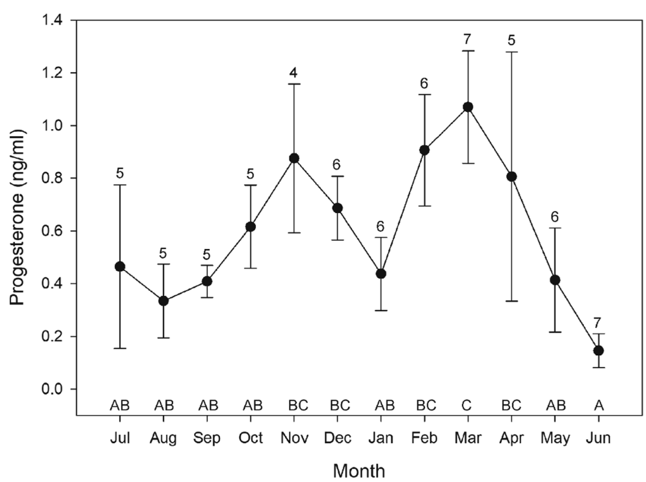

Fig. 4. Mean monthly plasma progesterone concentrations from 65 wild female *Gopherus polyphemus* samples collected from July 2016 through June 2017. Vertical bars represent ± 1SE. Letters indicate significant differences ($p < 0.05$) as indicated by Tukey-Kramer *post hoc* comparisons. Numbers above data points indicate sample size.

(Fig. 3). The monthly concentrations of progesterone also varied through the study period ($F_{11,64} = 3.29$, $P = 0.002$) (Fig. 4). Progesterone increased in October and November (mean 0.9 ng/ml ± 0.2) before decreasing back to minimal concentrations in January (mean 0.3 ng/ml ± 0.06). The concentration increased again in February with a peak in March (mean 1.1 ng/ml ± 0.21), before returning to minimal concentrations from May through September (mean 0.3 ng/ml ± 0.1).

4. Discussion

We present the first year-round reproductive hormone concentrations for the gopher tortoise to explore the reproductive physiology of a species with a natural range that exposes populations to differing environmental conditions. Male testosterone concentrations were elevated from September - December and at minimal levels from April to July, apart from a secondary peak during the month of June. Female testosterone and estradiol concentrations were highest from August to February, and lowest from May to July. Female progesterone concentrations increased in late fall and peaked during the month of March. These data suggest this population has an extended breeding season from fall through spring with mating likely occurring from September through March, and females depositing eggs during a winter to spring nesting season. This pattern differs from what is reported for gopher tortoises in the northern portion of their range where they mate from spring to early fall, brumate through the winter, and then deposit a single nest in late spring (Butler and Hull, 1996; Epperson and Heise, 2003; Iverson, 1980; Johnson et al., 2007; Smith, 1995). The patterns reported in this study resemble the reproductive patterns described for tropical and sub-tropical chelonians with elevated hormone cycles through cooler months.

The testosterone concentrations in males increased in the late summer to a peak in early winter. This peak is likely associated with increased spermatogenic activity, since the production of sperm is dependent on the presence of testosterone and similar androgens in other species of turtles (Huot-Daubremont et al., 2003; Rostal et al., 1994). This pattern reflects a form of pre-nuptial spermatogenesis, with proliferation and maturation of spermatozoa beginning in the fall and lasting until early spring, with reduced gonadogenesis occurring during the summer months (Currylow et al., 2013). Tortoises in northern populations begin spermatogenic and testosterone patterns in early summer and continue into fall, with a break in reproductive activity from late fall through early spring while in hibernation (Ott et al., 2000; Taylor, 1982).

The low testosterone concentrations in males during the summer indicate an inactive or possibly semi-quiescent period during this time where spermatogenesis and testicular growth are limited, but the deposition of sperm into the epididymis continues (White and Murphy, 1973). An exception occurs in June where an unexpected spike in testosterone increased to near peak levels. This appears to be a brief resumption of spermatogenesis and gonadal proliferation, like what has been observed for painted turtles (Callard et al., 1976), box turtles (Currylow et al., 2013), and map turtles (Graham et al., 2015; Shelby et al., 2000). This pattern differs from what is known of the testosterone cycle in northern populations of gopher tortoises where Ott et al. (2000) documented a unimodal testosterone pattern corresponding to the summer months.

Testosterone, estradiol, and progesterone concentrations varied in females across the sampling period. Testosterone began increasing in late summer, reaching a peak in fall and winter, before slowly declining to minimal levels during the summer months. Testosterone is a precursor steroid to estrogens in many reproductive pathways, and is also linked to follicular growth (Owens, 1980). The pattern of testosterone is similar to that observed for estradiol in the same females, indicating testosterone may be actively aromatized into estradiol as the reproductive season progresses. The highest concentrations of estradiol were seen from late summer through the middle of winter. Estradiol is known to bind to receptors in the hepatocytes of oviparous reptiles, thus triggering the production of, and transport of, egg yolk proteins into ovarian follicles (Cree et al., 1991; Heck et al., 1997; Ho et al., 1982). Thus, vitellogenesis appears likely to occur from August to February, peaking in November with vitellogenic activity decreasing from March to July as estradiol concentrations decline. The pattern observed in this population suggests an extended reproductive season when compared to northern populations that exhibit high estradiol levels from August through October (Ott et al., 2000; Taylor, 1982). The extended period of high estradiol concentrations is similar to patterns in species such as *Malaclemys terrapin* (Donini et al., 2018) and *Sternotherus odoratus* (McPherson et al., 1982) which are known to produce two or more clutches during the reproductive season (Iverson and Meshaka, 2006; Seigel, 1980).

Progesterone is associated with the ovulation of ovarian follicles, and therefore will exhibit a short-term spike just before nesting in oviparous reptiles (Callard et al., 1978; Licht et al., 1982; Wibbels et al., 1992). In this population, progesterone increases in October and November, and again in February and March, with concentrations returning to low levels from April to September. This pattern indicates the tortoises may have an extended nesting season that may provide an opportunity for producing more than one clutch. This pattern resembles that of other tropical and sub-tropical chelonians such as *Deirochelys reticularia* (Buhlmann et al., 2009; Gibbons and Greene, 1978) and the Kinosternidae family (Mahmoud and Klicka, 1972; Morales-Verdeja and Vogt, 1997; Wilson et al., 1999).

Tortoises in northern populations are known to breed from spring through early fall when testosterone (male) and estradiol (female) concentrations are elevated, and gonadal activity is high (Landers et al., 1980). Summer and fall mating likely result in the female storing sperm through winter hibernation until eggs are released to the oviducts during late winter ovulation, as is the case in other temperate species (Gist and Congdon, 1998; Ott et al., 2000; Palmer et al., 1998). Male tortoises in this study were observed mounting females from October through March, indicating an extended breeding period through winter, or one that may occur year-round since courtship behaviors are observed in all months, and gonadal cycles would likely ensure consistent supply of sperm in the epididymides. Thus, this population may be nesting in the winter through spring, with the potential for additional nesting throughout the other months of the year. The presence of depredated nests and neonates in another south Florida population also suggest year-round reproductive activity (Moore et al., 2009). Ashton and Ashton (2008) reported seeing shelled eggs in two females during November, directly supporting the idea of an extended nesting season, similar to what we have observed in this population. Moore et al. (2009) also reported courtship behavior in every month of the year, as we have likewise observed in our own population.

Intraspecific variation in reproductive ecology and breeding cycles have been documented in other reptiles (i.e. Ballinger, 1979; Pianka, 1970), and are often explained as a response to temperature variation among populations (Adolph and Porter, 1993; Duvall et al., 1982). Reproduction in the western fence lizard varies as much as three months across populations exposed to a temperature cline across elevation gradients (Goldberg, 1974). Likewise, temperature typically serves as a proximate cue for initiating reproductive cycles in turtles (Mendonça, 1987), and thus provides a mechanism by which nesting periods are adjusted to time-periods that maximize offspring survivorship. Iverson (1977) determined that six species of temperate chelonians (Emydidae) in north Florida have continuous nesting cycles as opposed to a seasonal pattern in conspecifics from more northern populations. Interestingly, the gopher tortoise was reported to lay a single clutch of eggs between April and July based on prior studies (Arata, 1958; Iverson, 1977).

The cycle demonstrated by gopher tortoises in southwest Florida suggests these populations are following reproductive patterns similar to that of tropical species (Moll and Legler, 1971) where gonad recrudescence may be associated with decreasing temperatures, instead of increasing temperatures as is the case for temperate populations. The mild winter temperatures allow them to actively forage year-round without a winter dormancy period. Energy acquisition is determined by the rate at which food is harvested and processed (Congdon, 1989) and this ultimately influences the energy allocated to reproduction (Congdon and Tinkle, 1982; Jordan and Snell, 2002). The increased activity and energy acquisition is likely correlated with the size of their annual energy budget and consequently with the amount of energy that can be allocated to reproduction. In southern Florida, this may result in an extended nesting season that may allow some individuals to produce two sets of eggs. Additional observations are necessary to determine the extent of their nesting season, and if individuals produce two clutches in a single nesting season.

The reproductive hormone cycles presented in this paper indicate the reproductive ecology of gopher tortoise populations south of the freeze line varies significantly with that of populations from more northern portions of their range. It is not known if this pattern is an evolutionary response to selection favoring increased reproductive output, or a plastic response to warmer year-round temperatures. In any case, the differences in hormone cycles indicate a response to the subtropical environmental conditions animals in this portion of their range are exposed to. The warmer year-round conditions likely increase foraging opportunities and the size of their energy budget, and therefore provide an opportunity for extended breeding activities that may occur year-round. The variation of the gopher tortoise's life history needs to be considered when making management decisions to ensure the intraspecific diversity of the species is preserved. Gopher tortoise populations in southwest Florida were recently shown to be genetically divergent from other populations throughout the state, and included unique haplotypes only found in this region (Winters et al., 2017). We suggest gopher tortoise populations should be managed within regional units that are delineated to recognize their ecological and genetic differences.

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