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Seasonal Variation of Corticosterone Levels in *Graptemys flavimaculata*, an Imperiled Freshwater Turtle

Will Selman¹, Jodie M. Jawor², and Carl P. Qualls²

Currently, little is known about the seasonal variation of corticosterone (CORT) levels, either baseline or stress response, within freshwater turtles. We conducted a seasonal CORT study with a species of freshwater turtle, *Graptemys flavimaculata* (Yellow-blotched Sawback; family Emydidae), that is endemic to the Pascagoula River system of southeastern Mississippi. *Graptemys flavimaculata* is commonly observed while basking on deadwood snags, with us using basking traps and dip nets as active capture methods. We caught both male ($n = 60$) and female ($n = 49$) turtles during the months of April–October during 2007 and 2008. Immediately after capture, we collected an initial blood sample, confined the turtle for 35 minutes, and then took a second blood sample. Competitive binding radioimmunoassays were done to determine CORT levels. Time zero CORT levels for both sexes were generally lower than previously reported levels for other turtle species, likely due to the trapping methods used. By time 35 min, CORT levels were significantly elevated relative to initial levels. We did not detect seasonal differences in time zero CORT levels for males or females. However, seasonal differences in time 35 CORT levels for males were detected, with higher levels observed during summer months (July, August) relative to spring (April) and fall (October) months. Seasonal differences were also detected in time 35 CORT levels of females, with lower levels observed during July (nesting season) relative to September. This study provides novel information and insight into the differences in seasonal physiological demands for this species, and aquatic turtle species in general, while also providing probable connections of physiology to seasonal life history events.

WITHIN vertebrate taxa, the role of glucocorticoids is believed to be primarily for energetics and metabolic regulation, while elevated levels are associated with the stress response of individuals to an acute stressor (Sapolsky et al., 2000). This response allows organisms, in the short-term, to overcome the potential adverse impacts of a stressor by 1) increasing gluconeogenesis and muscle function, 2) redistributing the immune system, 3) inhibiting reproduction, 4) decreasing food digestion/metabolism, and 5) increasing sensory awareness (Sapolsky et al., 2000; Wingfield and Romero, 2001). However, if there is a chronic stressor that remains present and prolongs this response, it could be detrimental to the organism and impact a range of physiological functions, including altering levels of circulating steroid hormones (Licht, 1984; Licht et al., 1985; Moberg, 1985; Greenburg and Wingfield, 1987; Mahmoud et al., 1989; Romero and Reed, 2005), suppressing reproduction (sometimes completely), and detrimentally inhibiting immune system action (Apanius, 1998; Sapolsky et al., 2000; Wingfield and Romero, 2001). As such, having an understanding of glucocorticoid levels in an animal is important when considering physiology, behavior, or conservation questions.

Many vertebrate taxa also have the distinct ability to modulate the glucocorticoid stress response in regards to sex, reproductive state, body condition/health state, environmental conditions, and across different seasons (Moore and Jessop, 2003). Distinct seasonal differences occur primarily in response to associated daily, seasonal, and/or energetically demanding life history events (Romero, 2002). Within avian fauna, possibly one of the best studied groups with respect to glucocorticoids, high corticosterone (CORT) levels have been associated with altered lipid deposition (Davenport et al., 1989; Dulloo et al., 1990), initiating migration (Silverin et al., 1989; Wingfield et al., 1990;

O'Reilly and Wingfield, 2003), increased foraging (Astheimer et al., 1992), and decreasing behavior associated with reproduction (Silverin, 1986; Wingfield and Silverin, 1986; Harvey and Hall, 1990). However, fewer studies have addressed reptilian taxa in an effort to determine the secretion patterns of CORT as it relates to seasonal events. Most studies with reptilian taxa have found that elevated CORT levels are associated with reproductive activity (Manzo et al., 1994; Wilson and Wingfield, 1992; Tyrrell and Cree, 1998; Jessop, 2001) or food consumption, fat deposition, and growth (Whittier et al., 1987; Girling and Cree, 1995). Several studies also suggested better correlations of CORT to activity level (Tyrrell and Cree, 1998), body condition (Dunlap and Wingfield, 1995), environmental temperatures (Dunlap and Wingfield, 1995; Tyrrell and Cree, 1998), or populations living near the periphery of their range (Dunlap and Wingfield, 1995). Conversely, several studies have found little correlation with CORT levels and any seasonal aspects (Grassman and Hess, 1992; Dunlap and Wingfield, 1995; Tyrrell and Cree, 1998). In summary, most studies within reptilian taxa suggest that 1) CORT plays a distinct role during times of naturally occurring high energy demand and 2) that elevated CORT plays a supporting role in mitigating stress in reptiles.

Even though turtles (order Testudines) are an ecologically well-studied reptilian group (i.e., distribution, reproductive biology, thermal ecology; Ernst and Lovich, 2009), very little is known about the stress response of turtles and the seasonal role of CORT in these species. Of the few studies to date in turtles, most have focused on two under-represented, North American taxonomic groups: sea turtles (families Cheloniidae and Dermochylidae: six species; Gregory et al., 1996; Jessop et al., 1999a, 1999b; Gregory and Schmid, 2001) and tortoises (family Testudinidae: three species; Ott et al., 2000; Lance et al., 2001; Kahn et al.,

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2007). Very few studies have focused on the largest group of North American turtles, the aquatic/semi-aquatic turtles of the family Emydidae (32 species; Cash et al., 1997; Cash and Holberton, 2005).

Turtles within the genus *Graptemys* (family Emydidae), commonly referred to as map turtles or sawbacks, are primarily found in rivers, streams, bayous, or oxbow lakes that drain into the Gulf of Mexico (Ernst and Lovich, 2009). Additionally, many of the 14 species of *Graptemys* are considered species of concern due to loss/decline of habitat and collection for the pet trade (Buhlmann et al., 2008; Lindeman, in press). The Yellow-blotched Sawback *Graptemys flavimaculata* is a small (males to 10.8 cm plastron length [PL], 225 g; females to 21.6 cm PL, 1675 g), riverine turtle that is endemic to the Pascagoula River and its tributaries of southeastern Mississippi, U.S.A. (Selman and Qualls, 2009; Selman and Jones, 2011). Observed population declines in the 1980s led to federal listing of this species as Threatened in 1991 (U.S. Fish and Wildlife Service, 1991). Following this ruling, several studies were initiated to determine the life history and ecology of the species (for review see Selman and Jones, 2011), as well as studies on steroid hormone levels (Shelby et al., 2000; Shelby and Mendonça, 2001).

Since *G. flavimaculata* is a highly aquatic turtle with most activity occurring underwater, we know little about the timing of mating and mating behaviors. Studies with related species have observed mating during the spring (Jones and Hartfield, 1990) and fall (Shealy, 1976), with females in the latter case storing sperm until the following nesting season. Female choice is believed to be the reproductive selective force in this species (Berry and Shine, 1980), with male competition or territoriality unlikely in this scenario. One histological study attempted to determine female gonadal activity in *G. flavimaculata* (Seigel and Brauman, 1994), but no reproductively mature females existed in the museum collections examined, with no further collections likely due to the Threatened ESA status of *G. flavimaculata*. Later studies on sex steroids (Shelby et al., 2000) indicated that male testosterone (T) levels in *Graptemys flavimaculata* were elevated during fall months of September and October, and slightly elevated during April, likely due to the onset of spermiogenesis/spermatogenesis and mating, respectively. Females exhibited estradiol (E₂) peaks during the spring in May and June, which coincided with the presence of preovulatory follicles determined via ultrasound (Shelby et al., 2000).

Due to the paucity of information relating to the seasonality of CORT cycles in this species, and freshwater turtles in general, we sought to determine multiple aspects of the CORT cycle. We hypothesize that 1) *G. flavimaculata* will elicit a significant, short-term CORT stress response, 2) circulating baseline levels of CORT will vary throughout the season in male/female *G. flavimaculata*, 3) the CORT stress response will vary throughout the season in male/female *G. flavimaculata*, and 4) body size or condition will impact baseline or stress response CORT levels.

MATERIALS AND METHODS

Study site.—The study site was a 4 km river stretch on the Leaf River (Forrest County, Mississippi, USA), a major tributary of the Pascagoula River system of southeastern Mississippi. This site was free of excessive human recreational boating (only nine boats observed in 186.1 hrs;

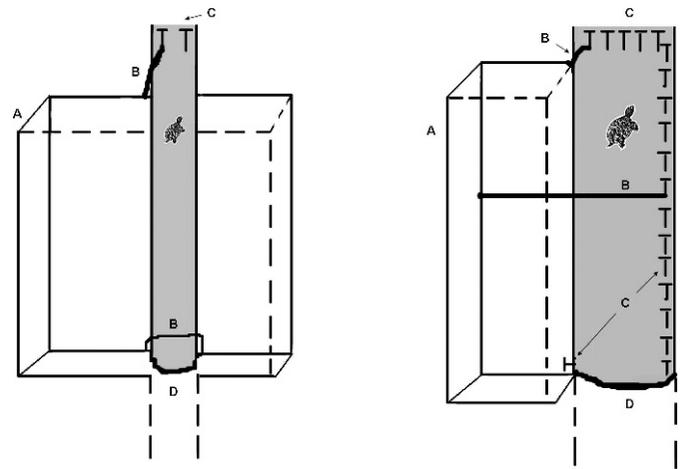


Fig. 1. Basking trap design for this study from an aerial view. The basking traps (A) are attached to the branch (at left) or log (at right) with cotton twine (B) and nails (C) below the surface of the water (D).

Selman and Qualls, 2011) that could potentially alter baseline and maximal CORT levels. The Leaf River is a medium-sized river (approx. 30 m wide) and the habitat is typical of a Gulf Coastal Plain river with alternating sandbar/cutbank sections, abundant submergent/emergent deadwood, and a sandy/gravel substrate. This study site was also chosen because earlier surveys had noted substantial numbers of *G. flavimaculata* in this area (Selman and Qualls, 2009).

Sample collection.—Turtles were captured by submerging open-topped, basking traps made of $\frac{3}{4}$ " PVC coated crawfish wire from turtle basking structures (Fig. 1) or by swimming and netting basking/swimming turtles during low river levels. Following basking trap placement, we would quickly approach the trap by motorized boat if a turtle was basking on the trap log. The boat approach would startle the basking turtle into the trap; we would only bleed a turtle if we saw it enter our basking trap/dip net and were able to bleed it within five minutes of capture, thus ensuring that we received a true baseline CORT value (Romero and Reed, 2005). When possible, blood samples were collected from five adult males and females per month from April to October in 2007 and 2008. The sex could be reliably determined due to extreme sexual dimorphism exhibited in this species, with the presumption that males had longer foreclaws and longer pre-anal tail length relative to females (Selman and Jones, 2011). We did not sample turtles during the winter months of November to March due to the inability to catch sufficient numbers of turtles because of lower basking frequencies during these months (Moore and Seigel, 2006). For a more detailed description of trapping methods see Selman and Qualls (2009).

Immediately following capture, 1 mL of blood was collected from female *G. flavimaculata* and 0.6–0.75 mL from males from the coccygeal vein using a heparinized 1 mL syringe and a 26½-gauge needle; smaller blood samples were taken from males due to their smaller size relative to females. Following this blood sample collection (hereafter referred to as baseline) and measurements of plastron length and body mass, individuals were placed into a 18.9-liter bucket (5 gallon) with approximately 5 cm of river water to prevent overheating, especially during the summer months.

The bucket was covered with a bucket lid, and we took another 0.1 mL blood sample 35 minutes after the initial sample collection (hereafter referred to as time 35); we considered the “stress response” as the difference between time 35 and time zero samples. Blood samples were stored on ice for 4 to 6 hours, and then centrifuged. Plasma and blood cells were separated, and then frozen at -20°C . Due to field conditions, samples were sometimes placed on dry ice following plasma/blood cell separation before entering a freezer.

Corticosterone assay.—Plasma CORT was measured using a direct radioimmunoassay (RIA; Wingfield and Farner, 1975; Ball and Wingfield, 1987; Ketterson et al., 1991). Approximately 2000 CPM of $\text{H}^3\text{-CORT}$ (PerkinElmer Life and Analytical Sciences, MA) was added to samples for later determination of extraction efficiency and tritiated samples were incubated overnight. We attempted to use a plasma sample volume of 50 μL in all analyses, but smaller volumes were used if we only had a small initial sample; RIA protocol can accommodate volumes this small (J. C. Wingfield, pers. comm.). Samples were extracted three times using 4.5 ml of diethyl ether per extraction. Following extraction and drying of samples under nitrogen gas, samples were reconstituted in 1 ml phosphate buffered saline solution with gelatin. Reconstituted samples were run in 100 μL duplicates in a RIA using radiolabeled CORT and an antibody specific to CORT (antibody sensitivity = 0.5 pg/tube; Fitzgerald Industries International, Inc., MA). Extraction efficiency (recovery) was based on a third aliquot from reconstituted samples and averaged 77%. Levels of CORT were determined from a nine point standard curve. Intra- and inter-assay variations were determined from a series of five known concentration standards per assay (i.e., quality controls) run in conjunction with the samples. Assays ($n = 12$) included both males and females, and both plasma samples for each individual (time zero, 35) were analyzed in the same assay. Intra-assay variation was 11.16%, inter-assay variation was 29%, and the lower detection limit of the RIAs was estimated at 0.0035 ng/ml. Due to the large number of assays and large interassay variation, a correction factor was applied to each sample. To calculate our correction factor, the “grand” mean of all assay standards was divided by the individual assay standard mean. Individual sample values were then multiplied by our correction factor (Jawor et al., 2006, 2007; McGlothlin et al., 2010).

Many samples fell below the detection limits for the assay (42 of 218 samples) and this was particularly evident for baseline samples (36 of 109). For our statistical analyses, these individual values were considered as the lowest detectability of the assay (0.0035 ng/mL) to provide more power to the analysis and more closely approach the “real” mean.

Statistical analysis.—Since we had small sample sizes for each month and no samples for some months in each year, we were unable to accurately assess differences across years. Therefore, we combined 2007 and 2008 data for each month for the following tests. To assess whether containment was effective in eliciting a stress response in this species, we used a nonparametric Wilcoxon Ranked Sum test to determine if baseline levels and time 35 CORT levels were the same. Males and females were analyzed separately. Due to the non-independence of sample points (i.e., collecting time zero

and time 35 blood samples from same individual), we conducted separate repeated measures ANOVAs for males and females. The selected model included CORT values as the dependent variable, month as a fixed factor (April–October), time of blood sample as a fixed factor (time zero or time 35), a time by month interaction, and individual turtle as a random effect (block) within the model. If there was a significant difference, a Tukey-Kramer *post hoc* analysis was done to delineate differences across months.

Due to the inability to transform time zero CORT levels to meet parametric assumptions for a linear regression test, we were unable to determine if time zero CORT levels or body condition were correlated. However, stress response data met parametric assumptions and a linear regression was performed to determine if stress response was correlated to 1) body size (plastron length) and 2) body condition. To determine body condition, we performed a linear regression of body mass by plastron length for males. Due to the log-linear nature of the female mass data (i.e., heteroscedasticity), we log transformed female mass to be more amenable to linear regression with plastron length. For both male and female regressions, we saved individual residuals as a measure of body condition. We analyzed sexes separately due to marked sexual size dimorphism since females attain twice the body length and ten times the body mass of males (Selman and Jones, 2011). For all statistical analyses, our significance level was $P < 0.05$, and we used JMP 8.0 (SAS Institute Inc., Cary, NC, <http://www.jmp.com>) for all statistical analyses.

RESULTS

Characterization of the CORT stress response.—During 2007 and 2008, we captured and collected baseline and time 35 plasma CORT samples from 49 female and 60 male *G. flavimaculata* (sample sizes by month shown in Fig. 2). Mean baseline CORT levels for females across all months was 0.38 ng/mL (SE \pm 0.08; range: 0–2.28 ng/mL), whereas mean baseline CORT for males was 0.52 ng/mL (SE \pm 0.09; range: 0–2.97 ng/mL). Mean time 35 levels for females across sampling months was 3.82 ng/mL (SE \pm 0.32; range: 0–11.33 ng/mL) and mean time 35 levels for males was 4.54 ng/mL (SE \pm 0.41; range: 0–13.22 ng/mL). There was a significant difference between baseline and time 35 samples for both males ($\chi^2 = 66.30$, $df = 1$, $P < 0.0001$) and females ($\chi^2 = 62.48$, $df = 1$, $P < 0.0001$), indicating that a significant stress response was initiated within 35 minutes.

Seasonal comparisons of CORT.—For females, we found a significant difference by month ($F_{6,42} = 3.15$, $P = 0.031$) and by time ($F_{1,42} = 147.0$, $P < 0.0001$), but the interaction was not significant ($F_{6,42} = 2.19$, $P = 0.06$). Time 35 CORT values were greater in September and lower in July, but neither was different from the other months (Fig. 2). Further, all time 35 values were greater than time zero. For males, we found a significant time by month interaction ($F_{6,53} = 2.64$, $P = 0.03$). Time 35 levels were greater in July and August relative to April and October, but not when compared to May, June, and September (Fig. 2). Time zero values were not different across months.

Body size and condition and the CORT stress response.—Body size was not correlated to stress response levels in males ($F_{1,59} = 3.49$, $P = 0.07$, $R^2 = 0.05$) or females ($F_{1,48} = 0.43$, $P = 0.51$, $R^2 = 0.009$). Further, body condition was not

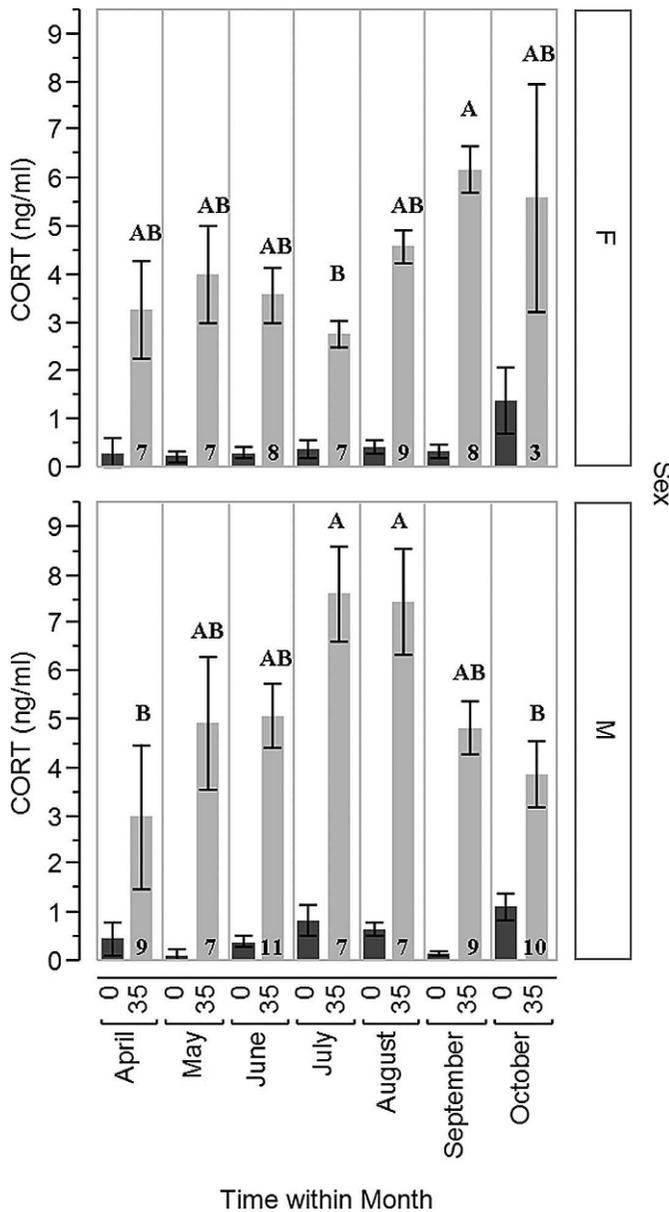


Fig. 2. Baseline (dark gray) and time 35 (light gray) CORT levels (ng/ml) for *Graptemys flavimaculata* at the Leaf River across sampling months for both sexes (females, top; males, bottom). Sample sizes for each month are indicated at the base of the stress response bar and significantly different CORT values by month are denoted by different letters. Time 35 samples are significantly greater than time zero samples for every month.

correlated to stress response in males ($F_{1,59} = 0.03$, $P = 0.87$, $R^2 = 0.0005$) or females ($F_{1,48} = 0.92$, $P = 0.34$, $R^2 = 0.019$).

DISCUSSION

Characterization of the CORT stress response and comparison across chelonian taxa.—Our baseline levels of CORT for male (0.52 ng/mL) and female (0.38 ng/mL) *G. flavimaculata* were generally lower than previous studies on chelonians. Cash and Holberton (2005) found that baseline CORT levels for aquatic slider turtles (*Trachemys scripta*) were 0.95 ng/mL (SD ± 0.187). Within the genus *Gopherus*, Lance et al. (2001) found that baseline CORT levels in desert tortoises (*Gopherus agassizii*) ranged from 0.20–4.08 ng/mL in females and 0.58–6.45 ng/mL for males, whereas Kahn et al. (2007) found the

highest reported baseline levels for turtles in gopher tortoises (*Gopherus polyphemus*; mean: 7.10 ng/mL, SE ± 1.80). Gregory et al. (1996) found baseline levels for loggerhead sea turtles *Caretta caretta* to be 0.55 ng/mL (SD ± 0.15) for tangle net capture and 2.07 ng/mL (SD ± 0.35) for trawl net capture. For both males and females, most levels reported in other studies are at least a two-fold increase relative to the levels described in our study. One explanation for the lower levels observed in our study may be related to our capture technique of open-topped basking traps and dip nets, which was a rapid capture and immediate sampling technique relative to other passive sampling techniques used in previous studies such as hoop nets, trawl nets, and Tomahawk live traps. Our trapping method ensured that the individuals we captured were not restrained for any time within a net, or an unknown amount of time spent in the trap, and could be bled almost immediately for more representative baseline CORT samples than animals that were trapped with other methods.

The stress response levels of *Graptemys flavimaculata* are significant for a small, chelonian species. Similar to our results, Gregory and Schmid (2001; Kemp's Ridley Sea Turtle, *Lepidochelys kempii*) and Cash et al. (1997; Red-Eared Slider turtle, *T. scripta*) found significant increases in CORT levels by 30 minutes after "time zero." Gregory and Schmid (2001) found much higher baseline (mean = 6.16 ± 2.31 ng/mL) and time 30 CORT levels (mean = 13.08 ± 3.53 ng/mL) relative to our study. Our CORT levels likely cannot be directly compared to either study due to the differences in trapping technique used. Gregory and Schmid (2001) used entanglement nets to capture sea turtles, while Cash et al. (1997) trapped individuals using a hoop net; individuals were bled within 15 minutes and 15–31 hours, respectively, for the "time zero" blood sample. Conversely, other CORT related turtle studies have found a more prolonged CORT response relative to our study. Within loggerhead sea turtles, *C. caretta*, Gregory et al. (1996) found that a significant CORT response did not occur until at least one hour post-capture.

Seasonal variation in baseline CORT levels.—Baseline CORT in females was not significantly different across months, but the mean CORT level values of our small sample size during October ($n = 3$) were slightly higher than all other months. The levels of circulating CORT (baseline) during this month may be associated with mobilizing and devoting more energy stores to autumn follicular development (Selman et al., 2009), as exhibited in another aquatic turtle species, *Sternotherus odoratus* (Mendonça and Licht, 1986). Further, as observed in other species of *Graptemys* (Shealy, 1976), this may be associated with fall mating, when baseline CORT levels are generally higher for previously studied chelonians (Romero, 2002). This would need to be confirmed in future studies with additional samples for this month and possibly for November.

Similar to females, there was not a significant difference in baseline CORT in males across months. However, we observed slightly higher mean CORT levels during the spring which may be associated with timing of mating (Lahanas, 1982). For most turtle species, the highest seasonal levels of baseline CORT have been recorded during the mating season (reviewed in Romero, 2002). During the fall, spermiogenesis is occurring (Shelby et al., 2000) and this process is likely energetically demanding. During this

time, there are also higher levels of circulating testosterone (Shelby et al., 2000). The slightly higher mean CORT levels during October may be associated with mobilizing and allocating more energy resources toward spermiogenesis, mating, or to help mitigate stresses associated with maintaining higher levels of testosterone; this would need to be confirmed in future studies.

Seasonal variation in elevated CORT levels.—Time 35 CORT for females was significantly lower in July and higher in September, with the seasonal trend appearing to be bimodal and, potentially, biologically relevant. It may be possible that the stress response is dampened during the month of July, which could be a response to the nesting season (i.e., oviposition) that occurs in this population from the middle of May through late July (W. Selman, unpubl. data); the timing of nesting is similar to another previously studied population of *G. flavimaculata* from the lower Pascagoula River (Horne et al., 2003). One explanation for this dampened stress response is that the nesting season could be the most stressful period for females relative to other months, possibly due to increased energetic demands associated with the finality of yolking ovarian follicles and associated nesting activities (Shelby et al., 2000; Horne et al., 2003). It may also be a physiological adjustment by females to avoid introducing detrimental levels of CORT into egg yolk that would compromise offspring fitness, as has been described in several avian and reptile species (Cree et al., 2003; Meylan and Clobert, 2004; Saino et al., 2005), or having elevated CORT levels interfere with nesting behavior. This would need to be confirmed in future studies by collecting larger sample sizes for these months and more accurately documenting reproductive status via ultrasound or x-ray.

The unimodal stress response of males throughout the season is evident, with significantly higher levels during the summer months of July and August, and lower time 35 CORT levels during spring and fall months. It is likely that mating occurs in the spring or fall, as copulation has been observed (*Graptemys oculifera*, Jones and Hartfield, 1990) or histological methods have determined this in other closely related taxa (*Graptemys nigrinoda delticola*, Lahanas, 1982; *Graptemys oculifera*, Kofron, 1991; *Graptemys ernsti*, Shealy, 1976). Along with mating, it is unclear if there is direct male competition or male combat, which would likely further exacerbate the physiological demands for males during this time. We presume that during the spring there are 1) likely fewer resources available for males to mount a significant stress response or 2) the CORT response is suppressed due to potential negative interaction it may have on T levels (Romero, 2002). By summer, males can mount a significantly higher CORT response, and we presume this is possible due to this being the least demanding period for males throughout the year or due to higher body temperatures as previously discussed. The lower stress response levels during the fall indicate that the stress response is limited during this period. This response may be similar to migratory birds, where excess CORT levels may induce protein/muscle catabolism (Holberton et al., 1996; Holberton, 1999; Long and Holberton, 2004); in our case, associated protein catabolism may be detrimental to sperm quality and/or sperm quantity during spermatogenesis.

One aspect of the seasonal cycle that was not considered during this study was the winter months of November

through March. In more northern latitudes, individuals may spend more than half of their lives overwintering, which is physiologically stressful (Ultsch, 2006). *Graptemys flavimaculata* does not hibernate during the winter months, but does exhibit seasonal movements toward favorable wintering habitat with lower flow rates and areas that are less frequently flooded (Jones, 1996). Even though they do exhibit some low-level activity during the winter, we could not capture sufficient numbers of individuals to collect blood samples during these months due to the lower tendency of individuals to bask (Moore and Seigel, 2006).

Body condition and the CORT stress response.—We found no correlation of the CORT stress response to either body size or body condition for either males or females. The role that body condition plays in the CORT stress response is quite variable, with some species having significantly correlated body condition and CORT stress responses (*Sceloporus occidentalis*, Dunlap and Wingfield, 1995; *Chelonia mydas*, Jessop et al., 2002; *Crocodylus johnstoni*, Jessop et al., 2003), while there is no correlation in other species (*T. scripta*, Cash et al., 1997; *Eretmochelys imbricata*, Jessop et al., 2004). Moore and Jessop (2003) noted that it is unclear why body condition is correlated to the CORT stress response with some reptile species, while it is not in others. Clearly, this is an area that demands further research studies with reptilian species.

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