

Research paper

Characterization of seasonal reproductive and stress steroid hormones in wild Radiated Tortoises, *Astrochelys radiata*



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ABSTRACT

The critically endangered Radiated Tortoise (*Astrochelys radiata*) is endemic to the southern coastlines of Madagascar. Once common, wild populations of this tortoise have undergone dramatic declines in recent years. Although there have been studies documenting reproductive activities, reproductive physiological parameters are unknown yet may be crucial in the recovery of the species. Over four research seasons in remote field locations native to *A. radiata*, we surveyed for, radio-tracked, and sampled wild, free ranging tortoises. We sampled and measured stress and reproductive parameters (corticosterone [CORT], testosterone [T], estradiol-17 β [E2], and progesterone [P]) in 311 plasma samples from 203 wild *A. radiata*, capturing their active period. Generally, hormone concentrations were associated with body condition, temperature, and humidity. There was wide variation in CORT that varied monthly and by group. Juvenile tortoises maintained more than twice the mean basal CORT concentrations than either adult sex, with the most dramatic distinctions in the middle of the wet season. For adult sex hormones, the last months of the dry season and into the wet season when ground humidities are low and just begin to rise prior to temperature declines, male T concentrations gradually increased to a peak before returning to near undetectable values into the dry season. We had limited data for T concentrations in females, but found average T concentrations were much lower than in males and positively correlated with larger female home range sizes. For female hormone cycles, E2 also peaked in the early 1/3 of the wet season along with male T, and was followed by an uptick in P which correlates to the putative ovulatory cycle. Females tracked over four years showed variation in patterns of P, indicating that number and frequency of clutches vary. Our results suggest that 1) there is high species plasticity in response to stress; 2) *A. radiata* reproductive cycling is somewhat dissociated with courtship timing and is instead triggered by environmental cues; and 3) individual female reproductive output is irregular. This study is one of the first to document and describe multi-year seasonal stress and reproductive hormones in a free-ranging Malagasy chelonian. These data may be used to identify key high-production habitats for conservation, and aide in captive management and reproduction in assurance colonies for species health and survival.

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1. Introduction

Understanding the natural variation in stress and reproductive physiology is an important tool for assessing the health of wild animal populations (Balestri et al., 2014; Sapolsky, 2005; Wikelski and Cooke, 2006). For many animals, reproduction is seasonal with

gonadal recrudescence and recesses that fluctuate in concert with sex steroid hormones (Dayger and Lutterschmidt, 2016; Norris and Lopez, 2011; Shero et al., 2015). This endocrine control of reproductive state may or may not be associated with reproductive behaviors and timing, and is often linked to environmental cues (Licht, 1982; Mahmoud et al., 2011; McPherson et al., 1982; Sereau et al., 2010). For many reptiles, temperature is often the most important factor in stimulating the production of hormones and affects seasonal reproductive timing (Licht, 1982; Mahmoud and Licht, 1997; Mendonça, 1987; Ott et al., 2000; Schramm

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et al., 1999). Deviation from these expected seasonal fluctuations or basal stress concentrations can indicate the presence of endocrine disruptors, or stressors such as disease or limited resources and may lead to disruption of reproductive condition (Kitana et al., 2006; Kitana et al., 2007; Shelby-Walker et al., 2009; Wingfield et al., 2016). Compounding this, animals that are stressed may not have the physiological resources needed to reproduce and, at a population-level, this can have severe consequences on species survival (Moore and Miller, 1984; Salvante and Williams, 2003; Wingfield et al., 1998). As a group, chelonians are one of the most endangered animals and opportunities for monitoring natural populations are becoming increasingly rare.

Stress and reproductive state can generally be tracked through monitoring of the associated circulating steroid hormones. Corticosterone is the primary stress hormone in reptiles and birds and is often used to assess health and chronic stress of animals (Mason, 1998; Romero, 2004; Wasser et al., 1997; Wingfield et al., 1997). The primary sex steroid hormones include Testosterone, Estradiol, and Progesterone (Edwards and Jones, 2001; Taylor et al., 2004). Testosterone is positively associated with development of testicular tissue (spermatogenesis) in male chelonians, and may occur with mating (associative) or outside the mating period (dissociative; Callard et al., 1976; Crews, 1984; Licht, 1982; Mendonça, 1987). In females, estradiol-17 β (E2) stimulates vitellogenesis, and ovarian development can generally be associated with elevated concentrations of plasma E2, while ovulation is associated with a spike in progesterone (P) concentrations (Edwards and Jones, 2001; Rostal et al., 1998; Taylor et al., 2004). In most reptiles, plasma E2 and P remain low until the immediate preovulatory period, making any detectable concentrations a good indicator that an animal is going to ovulate. Because it is difficult to study the physiological factors that influence reproductive status and behavior in wild chelonians (Gans and Crews, 1992), few studies have described field hormone profiles (Huot-Daubremont et al., 2003; Shelby et al., 2000; Trápaga et al., 2000). However, these types of studies are especially important for declining species, as typical seasonal reproductive patterns of natural populations should be the standard to which we compare captive or recovery populations. Temporal changes in hormone concentrations, body condition, and environmental fluctuations relate to aspects of energy budgets, reproduction, and immune responses (Amo et al., 2007; Ewenson et al., 2001; Henen, 1997). Therefore, we can use natural fluctuations recorded in wild populations to evaluate and improve these same health parameters in recovery populations.

Madagascar is known for its high rates of species endemism (Wilmé et al., 2006), yet declines in species richness and habitat conversion threaten its biodiversity. Madagascar's endemic Radiated Tortoise (*Astrochelys radiata*) is on the IUCN Red List and listed as Critically Endangered by the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES, 2016; IUCN, 2016). The species is experiencing precipitous population declines across its native range due to habitat destruction and collection (both for human consumption [adults] and the pet trade [juveniles]; Auliya et al., 2016; IUCN, 2016; Rafeliarisoa et al., 2013; Walker et al., 2014). Thus, conservation organizations are undertaking efforts, both in-situ and ex-situ, to preserve sub-populations, maintain and reproduce individuals in captivity, and repatriate animals. Yet captive reproductive efforts have highly variable results with little basis (e.g., expected baselines) from which to troubleshoot (Behler and Iadecola, 1990). Considering these reproductive challenges coupled with the severe population declines (Rafeliarisoa et al., 2013; Walker et al., 2014), it is likely that many *A. radiata* sub-populations have or will become functionally extinct in the wild.

Some aspects and observations of *A. radiata* reproduction have been published (Auffenberg, 1978; Hammer, 2013; Hammer, 2015; Leuteritz and Ravolanaivo, 2005), but stress and reproductive physiology have not yet been fully investigated or related back to observations. Little is known about baseline stress, underlying reproductive mechanisms, and natural hormonal patterns of these animals. To better understand reproductive potential of *A. radiata*, detailed stress levels and reproductive hormone profiles correlated to environmental cues are necessary. Though there is some information on reproduction of *A. radiata*, there have yet to be any longitudinal studies examining seasonal endocrine cycles using repeatedly followed individuals, and it is unknown if this species is an associative or dissociative reproducer. In this paper we 1) investigate stress seasonality by examining basal corticosterone concentrations in wild, free-ranging *A. radiata*; 2) describe seasonal reproductive hormone (testosterone, estradiol, and progesterone) patterns; and 3) associate hormone concentrations with activity, behavior, and environmental parameters (temperature and humidity).

2. Materials and methods

2.1. Study area

We studied wild, free-ranging *A. radiata* across their native range in the dry spiny forest habitat of south-southwestern Madagascar, along approximately 300 km of coastline from the village of Befasy through to Faux Cap (Leuteritz et al., 2005). Dry spiny forest habitat is characterized by the dominant Didiereaceae and *Euphorbia* spp. with sandy soils and spiny brush or dwarf vegetative undergrowth (Fenn, 2003). Temperatures range between 17 and 34 °C year-round and annual rainfall is approximately 350 mm, concentrated during the wet season (generally December–April; Rasoma et al., 2013).

2.2. Data collection and sampling

We conducted fieldwork in extremely remote areas across southern Madagascar where *A. radiata* are known to occur during the months of October through May over years 2012–2015. In spanning these eight months we collected data in both wet and dry seasons and captured the active period (generally November–April; Leuteritz, 2002) and surrounding months over multiple years. To initially locate and sample study animals across their natural range, we conducted meandering transect surveys for wild, free-ranging *A. radiata* as part of another study (Walker et al., 2014) in February–April for two consecutive years (2012–2013). In addition to population-wide sampling and as part of a concurrent radiotelemetry study (Currylow et al., unpubl. data and Currylow et al., 2017b), a subset of animals were repeatedly sampled (≤ 8 sample events) from October through May 2012–2015, allowing us to track individual variation over time. We used the radiotelemetry data from that study to calculate home range size (m^2) estimates of 30 animals (13 male, 12 female, and 5 juvenile) using the Minimum Bounded Geometry tool in ArcGIS (ESRI, 2015) and to assess vagility correlates with hormone concentrations.

To minimize stress artefacts in the sample, blood was taken first, immediately upon disturbance of the animal. We collected ≤ 1 cc of blood from the subcarapacial sinus (Hernandez-Divers et al., 2002) using 23-g, 2.5 cm needle on a 1- or 3-cc syringe. We transferred, processed, and stored samples as previously described in Currylow et al. (2017a, b). Briefly, samples were transferred immediately to heparinized vacutainers and stored in a cooler bag during field surveys until returning to camp (usually ≤ 5 h).

where they were centrifuged at 3300 rpm for ≥ 5 min by solar-generated stored power. Plasma was pipetted to cryovials and stored in liquid nitrogen in the field until transferred to a -20°C freezer (usually ≤ 4 w). After the final season of fieldwork was complete in 2015, all samples were transported to the United States on dry ice where they were stored in a -80°C cryogenic freezer. After sampling, we recorded animal's pre-disturbance GPS capture location (UTM, WGS 84) and any courtship activity, immobilized the animal using a pedestal, and proceeded with animal processing including measuring body weight (g), straight carapace length (SCL; mm), air temperature at 1 m above ground ($^\circ\text{C}$), ground temperature at surface ($^\circ\text{C}$), and ground surface humidity (%). We took care to avoid eliciting a stress response prior to sample collection which has been reported detectable within 17 min (± 2 min) of disturbance in this species (Currylow et al., 2017b). Median time to blood collection after disturbance was 2 min (20 s–12 min) and the median total processing time for each animal was 8 min (2 min–35 min). Once processing was complete, the animal was immediately returned to the location of capture.

2.3. CORT ELISA

To quantify corticosterone concentrations in animal plasma samples, we used commercially available corticosterone (CORT) EIA kits (Cayman Chemical ACE™, Item No. 500655). This assay platform's antibodies have previously been validated for use in *A. radiata* plasma (Currylow et al., 2017b). To ensure quality control of our assay, each sample was run at least in duplicate with an average intra-assay CV = 3.34% and inter-assay CV = 9.1%.

2.4. Reproductive hormone RIA

We measured plasma concentrations of the sex hormones, testosterone (T), estradiol-17 β (E2), and progesterone (P) using radioimmunoassay (RIA) kits (Immuno-Biological Laboratories Inc. #KIR1709 [T], #KIP0629 [E2], and #KIP1458 [P]). Samples were run following kit protocols, measured at least in duplicate with intra-assay CV averaging 2.03–2.45% and inter-assay CVs = 6.9–11.1%. For each kit, pooled and serially diluted *A. radiata* plasma showed high parallelism to the standard curves (Fig. 1). Accuracy was assessed using recovery of known standard additions to pooled plasma with mean recoveries of $97.4 \pm 4.2\%$, $98.6 \pm 3.9\%$ and $101.2 \pm 3.6\%$ for T, E2 and P, respectively.

2.5. Statistical analyses

To calculate a standard measurement that represents body condition score (BCS) across the age classes, we regressed straight carapace length (mm) with animal weight (g), fit a second-degree polynomial line to the data (e.g., Mandimbihasina and Currylow, 2014), and saved the residuals (Schulte-Hostedde et al., 2005). Individuals with a positive BCS (= residual value) are above the average weight for their size, and negative BCS indicates low weight to size. The larger or more negative BCS, the more extreme the body condition. To investigate monthly changes in seasonal hormone concentrations and cycles, we used linear mixed models with the hormone as the dependent variable and month, sex, BCS, and home range size (m^2) as the fixed effects with animal ID as the random effect to account for the repeated measures. To detect significant differences at each level, model tests were followed by post hoc comparisons of least squares means Tukey-Kramer (or Student's *t*-pairwise where < 3 variables) comparisons. Model residuals passed visual assessments for normality and homoscedasticity. Significance was set at $P \leq 0.05$ for all tests. All statistical analyses were run in JMP Pro 12 (SAS Institute Inc., 2015).

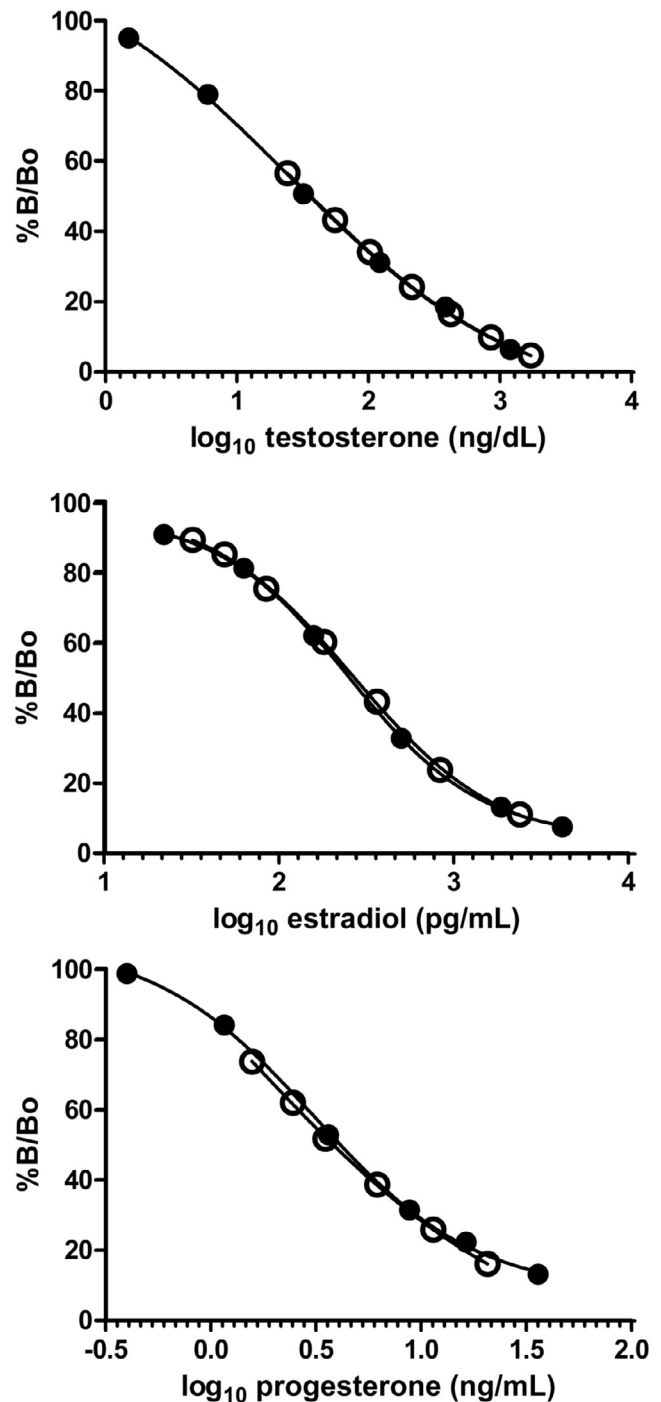


Fig. 1. Validation curves for plasma testosterone, estradiol, and progesterone in adult *Astrochelys radiata* overlain with standard curves for direct assessment of the degree of parallelism. Closed circles represent kit standards and open circles represent pooled and serially diluted *A. radiata* samples.

3. Results

Though we aimed to collect equal numbers of samples over the months and between the sexes, extreme and isolated field conditions (both in physically sampling animals and sample processing and storage challenges) prohibited a perfect balanced study design. As a result, we sampled a total of 203 (88 adult male, 84 adult female, and 31 unsexable juvenile) wild, free-ranging individual *A. radiata* from October through May from 2012–2015. From those individuals, some animals were sampled up to eight times over our

four-year study period, yielding a total of 311 useable plasma samples (139 adult male, 133 adult female, 39 juvenile) for CORT ($n = 310$), T ($n = 154$), E2 ($n = 131$), and/or P ($n = 129$) analyses. To best distinguish reproductive hormone concentrations between groups, this study did not include any sub-adult individuals that were close to maturation or early in their adulthood (~ 180 – 215 mm SCL). Described here are only animals ranging in size and weight from 243–408 mm/2900–12,500 g (males), 216–352 mm/1700–8500 g (females), and 55–178 mm/40–1300 g (juveniles). We report mean and median hormone concentration values \pm SE or provide a range in actual values for comparison to other studies.

3.1. Stress

We measured high individual variation from 310 samples in basal CORT concentrations (range = 14–68,374 pg/mL), and all but a single male at the upper end of the range yielded CORT concentrations under 11,000 pg/mL. When that outlier was excluded, we found differences between adults and juveniles ($F_{2,201.2} = 5.736$, $P = 0.0038$). Juveniles exhibited overall higher CORT concentrations (mean = 1921 pg/mL, median = 896 pg/mL, range = 91–10,534 pg/mL) than either adult males (mean = 841 pg/mL, median = 341 pg/mL, range = 14–7980 pg/mL) or females (mean = 782 pg/mL, median = 423 pg/mL, range = 35–9827 pg/mL). Female CORT concentrations were least variable over time, and only adult males exhibited a significant temporal change where March and April differed significantly between each other ($F_{6,113.1} = 2.343$, $P = 0.0360$; Fig. 2). Considering the conspicuous difference between mean and median CORT values amid all groups, however, further investigation beyond this study is warranted.

3.2. Reproductive hormones

We measured T in 137 male samples, and in 17 female samples for general comparison. There were significant differences in T between the sexes (mean male T = 203 ± 334 ng/dL, female 11 ± 24 ng/dL; $F_{2,146.5} = 3.117$, $P = 0.0472$). Male T differed by season ($F_{1,132.9} = 10.542$, $P = 0.0015$) and by month ($F_{6,125.7} = 13.668$, $P < 0.0001$), gradually increasing just prior to the wet season (October = 444 ± 94 ng/dL and November 603 ± 66 ng/dL) and peaking in January (862 ± 153 ng/dL) before it decreased dramatically into

March (60 ± 33 ng/dL) and remained low into the dry season (Fig. 3). There was no T difference between males that were tracked and those which were randomly encountered ($P = 0.9526$). When we analyzed the T samples from the 15 males which were radio-tracked and sampled multiple times, no differences could be attributed to individual effects or year, suggesting the seasonal pattern seen in pooling all samples is representative for the species.

We analyzed female samples for E2 ($n = 131$) and P ($n = 129$). Hormone concentrations varied by month (E2: $F_{6,76.3} = 2.878$, $P = 0.0140$; $F_{4,114.5} = 2.482$, $P = 0.0477$). Post-hoc analyses revealed that females exhibited a significant uptick in E2 in January (2022 ± 389 pg/mL) that was distinct from October, November, and March (means range = 515–652; Fig. 3). Progesterone differed significantly by season ($F_{1,126.9} = 4.621$, $P = 0.0335$), but the high variation in values caused differences between months to be non-significant except between October (0.18 ± 0.73 ng/mL) and February (1.66 ± 0.47 ng/mL). We also detected that CORT affected E2 in February ($F_{1,14.4} = 16.872$, $P = 0.0010$) and P in March ($F_{1,58} = 8.088$, $P = 0.0061$), the months after these hormones peaked. When we analyzed the samples from the 12 females that were radio-tracked and sampled multiple times, there were no differences in P but differences were detected in E2 concentrations in February between the years ($F_{3,48} = 3.364$, $P = 0.0262$). Seasonal E2 trends in these data followed that of the whole population, suggesting that the difference in E2 between years represents difference in individual clutch production (i.e., females do not produce clutches every single year). This coupled with high variation in P suggests that our sampling regime was not frequent enough to detect the relatively brief spikes expected in P that are associated with multiple nesting events.

3.3. Hormones and animal metrics

Straight carapace length (SCL) was highly predictive of animal weight (g) for *A. radiata* ($r^2 = 0.9453$, $P < 0.0001$); therefore, we continued with the use of residuals as a measure of BCS. There were significant differences in monthly mean BCSs among adult males ($F_{6,74.8} = 2.259$, $P = 0.0467$) and females ($F_{6,81.8} = 5.468$, $P < 0.0001$), but no monthly changes in BCS for juveniles (Fig. 4). Both males and females exhibited the highest average BCS in April (male: 8223 ± 479 g wt., 343 ± 8 mm SCL; female: 6811 ± 439 g wt., 318 ± 9 mm SCL) while juveniles varied little over the sample period (674 ± 57 g wt., 140 ± 4 mm SCL; Fig. 4).

Adult male T was negatively associated with positive BCSs in October ($F_{1,5} = 8.805$, $P = 0.0313$). Adult female BCS had significant negative effects on P concentrations in October ($F_{1,111} = 15.411$, $P = 0.0002$) and positive effects in February ($F_{1,18} = 4.868$, $P = 0.0407$) and March ($F_{1,71} = 10.201$, $P = 0.0021$).

Adult females exhibited an inverse association between BCS and CORT in January ($F_{1,2} = 101.818$, $P = 0.0097$), at the time when E2 peaked, and just prior to the spike in P, indicating this change is associated with peak nesting events. Female P concentrations influence BCS ($F_{1,70.8} = 5.050$, $P = 0.0277$), and post hoc analysis showed that the largest influence was in November and April, and were associated with low and highest BCSs, respectively. Among the tracked females ($n = 7$), higher T was associated with larger home range size ($F_{1,5} = 11.924$, $P = 0.0182$).

3.4. Environmental factors

Environmental conditions at tortoise locations during sampling are shown in Fig. 3. Ground temperatures negatively affected CORT concentrations in juveniles ($F_{1,26.6} = 4.538$, $P = 0.0426$) and positively affected CORT in males ($F_{1,112.6} = 3.978$, $P = 0.0485$). We also detected positive associations between ground temperature and T

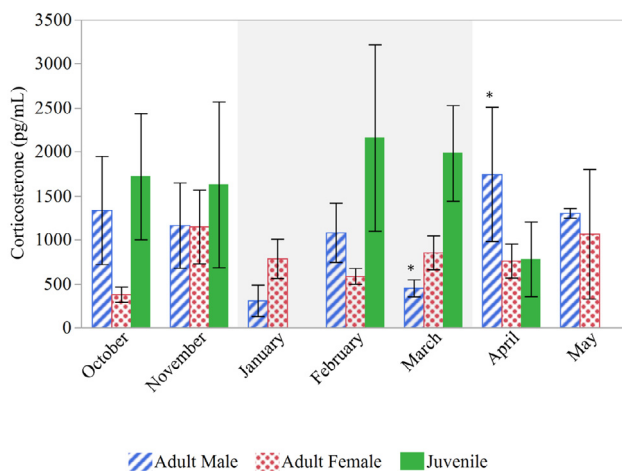


Fig. 2. Mean monthly corticosterone concentrations from 311 wild *Astrochelys radiata* plasma samples from 2012 to 2015. Shaded area represents general active/wet season. Asterisks represent monthly values which are significantly different from each other. Error bars represent 1 SE.

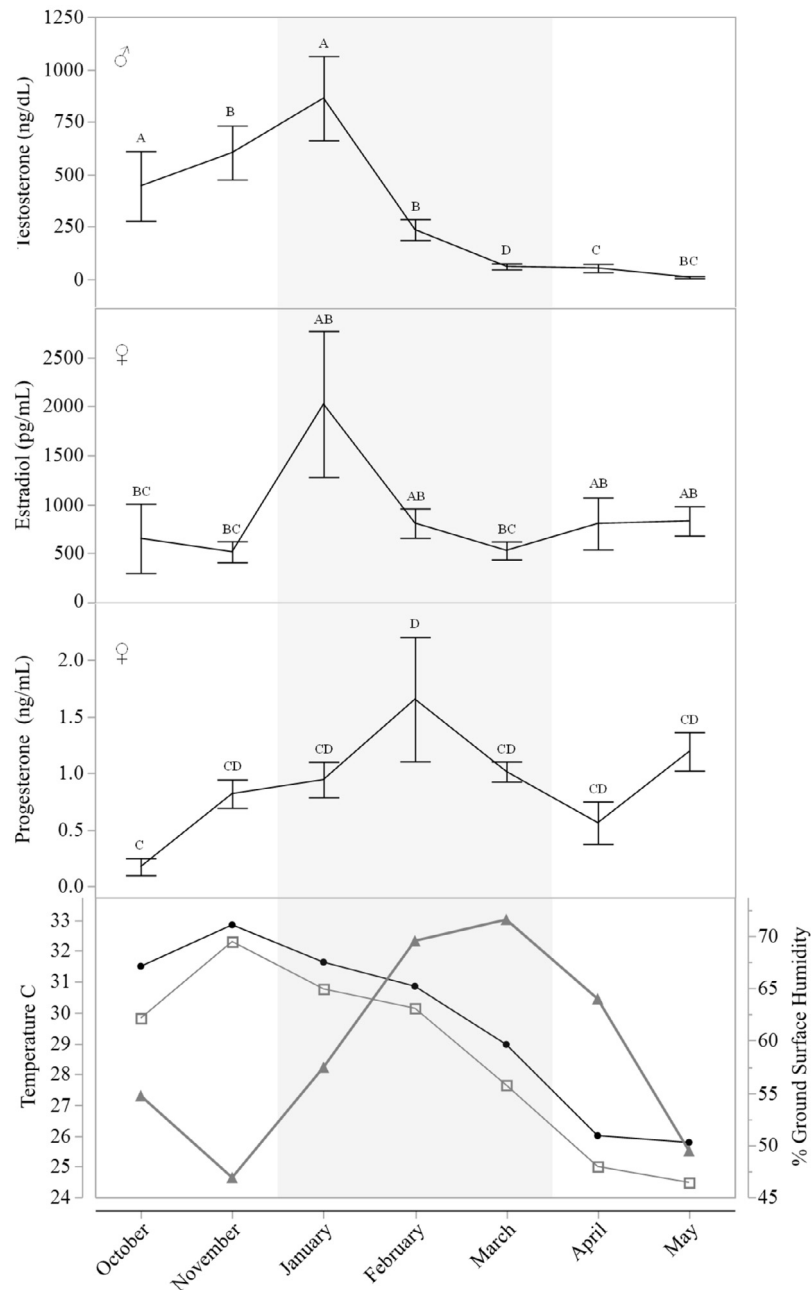


Fig. 3. Mean monthly plasma testosterone concentrations from 141 wild adult male *Astrochelys radiata* samples (top), mean monthly plasma estradiol and progesterone concentrations from 143 wild adult female samples (mid), and mean monthly environmental variables associated with *Astrochelys radiata* locations (bottom) in southern Madagascar from 2012 to 2015. Shaded column represents general active/wet season. *Top and mid:* monthly hormone means not connected by the same letter in each plot are significantly different; error bars represent 1 SE. *Bottom:* open grey squares = air temperatures in °C; solid black circles = ground temperatures in °C; solid grey triangles = % ground humidity.

concentrations in males ($F_{1,111} = 15.411$, $P = 0.0002$) and E2 in females ($F_{1,123} = 7.016$, $P = 0.0091$). Ground humidity negatively affected T in males ($F_{1,135} = 6.962$, $P = 0.0093$), and positively affected P concentrations in females ($F_{1,129} = 5.630$, $P = 0.0191$). Air temperature positively affected CORT in males ($F_{1,111.9} = 4.212$, $P = 0.0425$) and E concentrations in females ($F_{1,112} = 6.305$, $P = 0.0135$).

4. Discussion

We present the first empirical data (primary endocrine markers) to longitudinally describe the basal stress levels and

reproductive patterns of *A. radiata*. Seasonal patterns in hormone concentrations for each sex were associated with body condition, temperature, and humidity. Similar to other chelonian strategies, CORT concentrations were highest in juveniles *A. radiata* and adults exhibit semi-dissociative reproduction, where gonadal activity and courtship are temporally separated (Crews and Moore, 2005). A single, gradual rise in T was found in males prior to purported peak reproductive behaviors, whereas females showed a single strong peak of E2 (both in January), followed by a peak in P in February, which may have been associated with early nesting but not with peak nesting or multiple nests. The population-scale of this study, however, might be too broad of detail needed to identify the

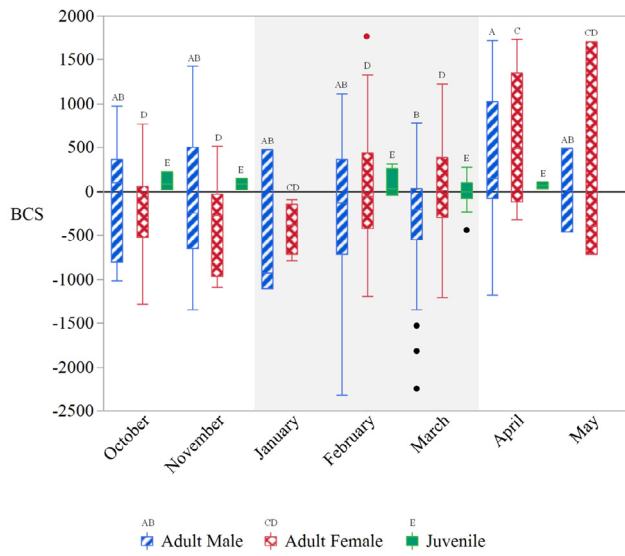


Fig. 4. Boxplots of body condition scores (BCS) of each group (adult males, adult females, and juveniles) by month for free-living *Astrochelys radiata* from southern Madagascar. Solid black circles are outlier points. Shaded area represents general active/wet season. Monthly boxplots within the same group and not connected by the same letter are significantly different. See Section 2.5 text for BCS calculation and description.

multiple nesting events seen in individuals. Environmental cues (ground humidity and air temperature) were the strongest predictors of increased production of sex steroid hormones. Hormone patterns suggested that female reproductive output is not annual as variation was seen in monthly sex hormone concentrations between individuals or over multiple years. Yet female T is positively correlated with home range size in the smaller subset of radio-tracked animals.

4.1. Stress

Consistent with other studies (Cabezas et al., 2013; Douxflis et al., 2011; Mason et al., 2013), the highest mean CORT concentrations were exhibited by juveniles, at more than twice the concentrations of the adults. The largest difference in CORT concentrations between the age classes was in the middle of the wet season and at the peak of activity for this species. Elevated CORT may be adaptive because it is associated with predator avoidance behaviors, learning and memory, and cautious animal personality types (McEwen and Wingfield, 2003; Moore and Jessop, 2003; Sapolsky et al., 2000; Thaker et al., 2009), and these associations are especially important for young animals actively learning their environment. Beyond the juveniles, there was great individual variation in CORT concentrations within this species. The broad distribution of CORT concentrations indicates the potential for confounding factors which have not been investigated here. In a recent study on a subset of wild *A. radiata*, the variation in basal CORT concentrations and the degree of individual stress responses were associated with a suite of longer-term, individual's traits such as home range sizes, BCSs, movement frequency, and activity (Currylow et al., 2017b). Also related to CORT and vagility, an experimental study of the semiaquatic turtle *Trachemys scripta* by Cash and Holberton (2005) subjected animals to artificially decreased habitat quality by slowly draining their residential ponds. The animals exhibited increased CORT concentrations, even more dramatically than a trap capture stress response, and triggered increased vagility in the animals (Cash and Holberton, 2005). Basal CORT concentrations may be used as an indicator of

how individuals react to their environment and how likely they may be to survive in stochastic conditions.

A positive correlation between sex hormones and CORT has been previously shown in tortoises (Lance et al., 2001; Schramm et al., 1999), and we could detect a similar interaction between CORT and sex hormones in females at certain times in the year. Our lack of further correlation could be because the data from this wild, free-ranging population were clouded by confounding variables we did not measure. It may be that this species is less affected by sex-related stressors compared to other species with strong mating behavior correlations. However, in a study of the closest sister taxa, *A. yniphora*, this trend did not appear to hold true (Currylow et al., 2017a). *Astrochelys radiata* breed readily in captivity, and the dissociation between CORT and sex hormones might occur because this species is evolutionarily better equipped to handle stressors, or to breed despite the presence of anthropogenic stressors (e.g., Polich, 2016; Winters et al., 2016). More research on the influence of CORT on reproduction in this and other chelonian species which vary in captive reproductive potential should be conducted.

4.2. Reproductive hormone patterns

Astrochelys radiata are active year-round, but become less active during the dry season (April–November) when rainfall is minimal and temperature fluctuations are less buffered (Hammer, 2015). Concordantly, we found that *A. radiata* hormone profiles reflected seasonality. We also found that much of the significant interactions in our *A. radiata* data were associated with the point of inversion between temperatures and humidity, which indicated the onset of the wet season. Just prior to this inversion when ground surface humidity begins to rise, but before air and ground temperatures decline, there is an increase in male T, increase in female E2, BCS is at its lowest, and mean adult CORT decreases for the duration of the season, all indicators of reproductive cycles as discussed below.

Male *A. radiata* exhibit a gradual uptick in T late in the dry season, culminating to a single peak early in the wet season. The especially long duration of high T suggests that *A. radiata* undergo gonadogenesis in preparation for mating and persistence of courtship. Though, a lack of a clear peak confounds the assignment of associative or dissociative breeders, perhaps classifying *A. radiata* as a combination of the two. Anatomical and endocrine studies on other male chelonians suggests that many are at least partially dissociated reproducers (where seasonal gonadal recrudescence is generally shifted temporally with mating behaviors; Callard et al., 1976; Crews, 1984; Mendonça, 1987). Alternatively, many male chelonians exhibit a dual peak in T, one near the beginning of the active period which does appear to play a facultative role in courtship activities, and another peak nearer or at the end of the mating period which does not correlate with courtship (Currylow et al., 2013; Kuchling et al., 1981; Rostal et al., 1994b; Sereau et al., 2010; Shelby et al., 2000). In early studies of *Sternotherus odoratus*, *Terrapene carolina*, and *T. ornata*, the testes of sacrificed animals were dissected and inspected, revealing early-season development prior to mating, mid-season recession, and a late-season recrudescence (Altland, 1951; Legler, 1960; Risley, 1938). It is thought that the second T peak enables the recrudescence of testicular tissues in preparation for the next reproductive season before environmental conditions (i.e., limited resources) become too unfavorable to support such growth. In equatorial habitats like Madagascar, the distinction between the active and inactive periods are less stark than temperate locations, and animals are often active year-round (though less so in the dry season; Rasoma et al., 2013; Currylow et al. unpubl. data). It could be that in *A. radiata*, a dual peak in T is not necessary because conditions are not so harsh

as to preclude regressed testes from developing just prior to the onset of mating period. Though, it is also possible because we only sampled surrounding the wet season, that another peak in T occurs outside of our sampling window. If that is the case, upregulating gonadal activity would still occur during the inactive period in this species. Considering that some species exhibit a dual peak in T and an associated recrudescence of testicular tissue into periods of inactivity, the single semi-dissociative T elevation we found should not be considered too unusual.

For female *A. radiata*, clutches may be produced multiple times in a year, with nesting generally occurring from January through June, peaking in April, with second and third clutches extending into later months (Hammer, 2015; Leuteritz and Ravolanaivo, 2005). Though equatorial chelonians generally have more frequent clutches per annum due to protracted seasons of favorable weather conditions, restricted access to resources may limit the number of clutches or reproductive output altogether (Iverson, 1992; Iverson et al., 1993). Over the whole population in the present study, females generally ovulated in February as evidenced by the peak in P just after we see a surge in E2 the previous month. At this scale, multiple clutches are likely not detected because our sampling regime was likely not frequent enough to detect the relatively brief spikes of P that are usually associated with multiple clutches (Blanvillain et al., 2011). It is worth noting, however, that the peaks we found in the female data do not correlate best with nesting event observations seen in wild animals in previous studies which describe peak nesting in April (Hammer, 2015; Leuteritz and Ravolanaivo, 2005). Endocrine research involving captive *A. radiata* would be useful in verifying a hormone-ovulation relationship in this species because reproductive condition and output can be closely tracked with ultrasound, radiography, and monitoring, then paired to E2 and P concentrations.

Though we could not detect multiple clutches in a season, we found endocrinological evidence that *A. radiata* females do not necessarily produce a clutch each year. Understanding that a pre-nesting surge in P can be particularly brief (sometimes lasting only 24-h; Licht, 1982), the range and temporal variation in our multiply sampled individuals suggests that the population-wide data likely reflect the inequality in annual reproductive output (frequency) between individuals. This is consistent with field reproductive studies in this species. In one study that radiographed wild *A. radiata* over a single season, the authors found that two of 12 females did not become gravid, while others produced 1–3 clutches that same season in the same habitat (Leuteritz and Ravolanaivo, 2005). Another found that only 10–35% of wild females nested any given year, with only two from a captive population laying in two consecutive years (Hammer, 2015). The number of clutches a female produces can also be latitudinally linked (Du et al., 2014), and such may be the case in *A. radiata*. In a study at the southeastern extent of the species' range Leuteritz (2002) reported *A. radiata* produce 1–3 clutches each season while a later study at the northwestern extent found that only a single individual out of 19 females laid more than a single clutch in a given year (Hammer, 2015).

4.3. Hormone effects and animals

Astrochelys radiata courtship and mating occur primarily in the wet season (December–March; Hammer, 2015; Leuteritz, 2002; Leuteritz and Ravolanaivo, 2005). Likewise, we made 12 observations of mating or courtship behaviors, all within the most active period (January–March). Previous studies have shown a link between T and courtship or aggression in other reptiles (Currylow et al., 2013; Moore and Marler, 1987; Sinervo et al., 2000; Sereau et al., 2010). We did not find a similar direct association here, though our sample size was quite small (4% of all adult

activity records). It has been suggested that although some species exhibit a dissociation between T and courtship behaviors, a late-season peak in T could still drive reproductive behaviors the following season (Licht, 1982).

Though in much lower mean concentrations than found in males, female's T was positively correlated with their home range size. Our results compliment another recent study in this species which investigated longer-term behavioral associations with CORT concentrations, where individuals with higher CORT and more dramatic stress response were animals that maintained larger home ranges (Currylow et al., 2017b). Yet, these findings contrast with the relationship of T in another chelonian, the Eastern Box Turtle (*T. carolina carolina*) where males with the highest levels of circulating T maintained smaller home ranges (Currylow et al., 2013). It may be that adult females in the current study were more explorative due to the increasing habitat degradation. Female T has been correlated with ovulation (Callard et al., 1978; Licht, 1982) and those with higher T might have been looking for nesting habitat. Appropriate nesting habitat may be more difficult to find in southern Madagascar where the human-wildlife interface is high. However, some chelonian species are not perturbed by human activity during nesting (Bowen and Janzen, 2008; Polich, 2016), and there is some evidence that *A. radiata* may prefer semi-disturbed habitats for nesting (Currylow et al., 2014). The role that T plays in the organization of behavior in this species is just beginning to be unraveled.

As expected, lower BCS in females correlated with probable nesting as indicated by the rise in P. Suitably, previous studies found that larger *A. radiata* females lay clutches of larger and heavier eggs (Hammer, 2013, 2015; Leuteritz and Ravolanaivo, 2005). Female BCS then recovered by April. For males, T negatively affected BCS, likely due to the refocus of resources from growth to courtship behaviors. In a study on the growth of young lizards, researchers found that increased T inhibited body growth, and proposed this as a mechanism for reaching sexual maturity more quickly (Cox and John-Alder, 2005).

The data presented here can represent baseline data and be used as a conservation tool for improving captive conditions and wild recovery efforts of this species. Recovery efforts often involve captive breeding and repatriation along with habitat preservation and protection. Besides using these data as captive condition guidelines and monitoring, further work can be done. For example, the use of T has proven useful in sexing sub-adult sea turtles (Wibbels et al., 1987) and has been shown to be 98% accurate for another tortoise species (Rostal et al., 1994a). Considering that male T was distinguishable from females, and that E2 was highest in females in January, testing sub-adults during peak season (January) may be a way to distinguish the sexes and ensure release of equal sex ratios into the wild from captive breeding programs.

We present the first data on circulating steroid hormone levels in wild adult and juvenile *A. radiata*. The primary stress hormone (CORT) is presented temporally by sex and age class, and sex hormones (T, E2, and P) are presented for adults. Hormone concentrations were influenced by body condition, activity, behaviors, and environmental conditions and offer important insights into the reproductive and behavioral ecology of *A. radiata*.

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