



Steroid hormones and aggression in female Galápagos marine iguanas

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Abstract

We studied steroid hormone patterns and aggression during breeding in female Galápagos marine iguanas (*Amblyrhynchus cristatus*). Females display vigorously towards courting males after copulating (female–male aggression), as well as fight for and defend nest sites against other females (female–female aggression). To understand the neuroendocrine basis of this aggressive behavior, we examined changes in testosterone (T), estradiol (E₂), corticosterone (CORT), and progesterone (P₄) during the mating and nesting periods, and then measured levels in nesting females captured during aggressive interactions. Testosterone reached maximal levels during the mating stage when female–male aggression was most common, and increased slightly, but significantly, during the nesting stage when female–female aggression was most common. However, fighting females had significantly lower T, but higher E₂ and P₄, than non-fighting females. It remains unclear whether these changes in hormone levels during aggressive interactions are a cause or a consequence of a change in behavior. Our results support the “challenge hypothesis”, but suggest that E₂ and/or P₄ may increase in response to aggressive challenges in females just as T does in males. Females may be rapidly aromatizing T to elevate circulating levels of E₂ during aggressive interactions. This hypothesis could explain why non-fighting females had slightly elevated baseline T, but extremely low E₂, during stages when aggressive interactions were most common. Although P₄ increased rapidly during aggressive encounters, it is unclear whether it acts directly to affect behavior, or indirectly via conversion to E₂. The rapid production and conversion of E₂ and P₄ may be an important mechanism underlying female aggression in vertebrates.

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Introduction

Although most studies of aggression in vertebrates focus on males, females are aggressive in a variety of social and reproductive contexts (Floody, 1983). Female aggression can be directed at both males (female–male aggression) or at other females (female–female aggression). Female–male aggression can be manifested in competition over mating (Carpenter, 1966) and protection of offspring (Hausfater and Hrdy, 1984), whereas female–female aggression occurs when females compete over resources (Gowaty and Wagner, 1988; Jaeger et al., 1982), compete over access to males (Hurly and Robertson, 1984; Slagsvold and Lifjeld, 1994; Summers, 1989; Walter and Trillmich, 1994), fight to

establish social rank (Walters and Seyfarth, 1987), and fight to defend territories (Carpenter, 1966; Eibl-Eibesfeldt, 1966; Rauch, 1988; Woodley and Moore, 1999a).

Despite its high occurrence in mammals, birds, and reptiles in a variety of social and reproductive contexts, female aggression is poorly understood from a behavioral neuroendocrine basis, particularly when compared to male aggression. Aggression in females seems to be at least partly mediated by steroid hormones. In territorial species, testosterone can play an important role in female territorial displays in birds (Cristol and Johnsen, 1994; Wingfield, 1994) and reptiles (Woodley and Moore, 1999a). In female mammals, testosterone may interact with other steroids to affect aggression in some species (Albert et al., 1992; Barkley and Goldman, 1977; Kapusta, 1998), but it has no effect in others (Gleason et al., 1979; Payne and Swanson, 1972). In many cases, however, it is unclear whether

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testosterone or its metabolite, estradiol, directly affects female aggression (Elekonich and Wingfield, 2000; Rhen et al., 1999). Estradiol promotes aggressive behavior in female reptiles (Woodley and Moore, 1999a) and may regulate it in pregnant and lactating mammals, but suppress it in sexually receptive mammals (Mayer and Rosenblatt, 1987). Similarly, progesterone may influence aggression in some contexts but suppress it in others (Davis and Marler, 2003; Erpino and Chappell, 1971; Meisel et al., 1988; Svare et al., 1986).

We chose to examine the relationship between steroid hormones and female aggression in a free-living lizard, the Galápagos marine iguana (*Amblyrinchus cristatus*), under natural conditions. Only a few studies have examined aggression directly in female lizards; in the mountain spiny lizard (*Sceloporus jarrovi*), testosterone and estradiol seem to promote female aggression, while corticosterone seems to suppress it (Woodley and Moore, 1999a; Woodley et al., 2000b). Marine iguanas make an ideal study system because breeding is relatively synchronous (Rubenstein and Wikelski, 2003) and females can be extremely aggressive during certain times of the year in two reproductive and social contexts (Carpenter, 1966). Specifically, female–male aggression over copulating occurs only during the mating period, whereas female–female aggression over nesting sites only occurs during the nesting period, approximately 4 weeks after copulations (Rauch, 1988; Wikelski, personal observations). Immediately after copulating, females change both morphologically and behaviorally to resemble males. Males of this lekking species defend territories during the mating season and use head-bob displays to court females. After copulating, females alter their skin color and resemble breeding males, gaining red patches on their flanks and bright green colors along their dorsal spines (Trillmich, 1983). Females also alter their behavior, elevating their posture and head-bobbing aggressively to courting males to signal that they have already copulated (female–male aggression) (see description of male courtship behavior in Carpenter, 1966). After mating has finished and females move to the nesting area, they aggressively try to steal or defend nest sites from other females because high quality nesting sites are limited (female–female aggression) (Boersma, 1983; Carpenter, 1966; Eibl-Eibesfeldt, 1966; Rauch, 1988; Rubenstein, personal observations). Females defend nest sites for a mean of 3 days (range of 0–9 days) before and a mean of 5 days (range of 0–16 days) after egg-laying (Rauch, 1988).

In this study, we examined baseline steroid levels in different reproductive stages and measured a variety of potentially important steroids related to female aggression over rapid time courses during aggressive interactions when the potentially subtle and short-lived elevations in steroid levels would be most visible. By doing so, we explicitly tested predictions of the “challenge hypothesis”, which explains patterns of seasonal testosterone variation in relation to levels of intrasexual aggression and competition

in males (Wingfield et al., 1990). Although there have been few tests of the challenge hypothesis in females, steroids other than testosterone (e.g., progesterone) seem to respond to aggressive challenges much as testosterone does in males (Davis and Marler, 2003).

The first step in understanding how hormones regulate and/or respond to female aggression in lizards is to examine how baseline patterns of sex steroids vary during the breeding season. Several studies have examined the seasonal changes in steroid hormones in female lizards (Amev and Whittier, 2000; Jones et al., 1997; Radder et al., 2001; Rhen et al., 2003; Shanbhag et al., 2001), but none have looked at iguanine lizards. We monitored female marine iguanas during the mating and nesting periods, measuring testosterone (T), estradiol (E₂), corticosterone (CORT), and progesterone (P₄), as well as two measures of body condition, hematocrit and a body condition index score (Laurie, 1989; Romero and Wikelski, 2001; Wikelski and Trillmich, 1997).

Next, we examined hormone levels during the mating and nesting stages when aggressive interactions are most common, as well as during actual aggressive interactions. Although we examined steroid levels and female–male aggression during the mating period, we focused more on female–female aggression during the nesting period because in most reptiles, T and E₂ are elevated (and possibly maximal) during the mating period and thus may not increase further (Cree et al., 1992; Edwards and Jones, 2001; Guillette et al., 1997; Hamann et al., 2002; Ott et al., 2000; Radder et al., 2001; Rhen et al., 2003; Saint-Girons et al., 1993; Taylor et al., 2004; Whittier et al., 1987). To test the challenge hypothesis in female marine iguanas, we compared (1) baseline steroid levels in three different mating stages, (2) baseline steroid levels in four different nesting stages, and (3) steroid hormones in females captured during aggressive interactions to non-fighting females caught digging and covering nests. We predicted that (1) baseline levels of T, E₂, and/or P₄ would be elevated during the post-copulation mating stage when females need to be most aggressive to fend off males attempting to copulate with them after they have already copulated; (2) baseline levels of T, E₂, and/or P₄ would be elevated during the nest digging and covering stages when females need to be most aggressive for nest site takeover or defense; and (3) levels of steroids directly related to aggression during interactions (or challenges) would be higher in fighting females than in non-fighting females.

Methods

Study site

This study was conducted on the island of Santa Fé (90° 02' W, 0° 50' S), one of the central islands in the Galápagos, from 11 November 1999 until 10 February 2000. Our study

site at Miedo Beach consisted of a ca. 75-m steep, rocky beach coastline with intertidal zone flats. Approximately 100 m inland from the beach was a sandy/dirt area where females nested. Females spent the entire pre-breeding (mid-October through early December) and mating periods (mid-December through mid-January) along the coastline. Individuals moved to the nesting beach for a period of 1–4 weeks during the nesting period (mid-January through February) (Carpenter, 1966; Rauch, 1988), and upon laying their eggs, returned to the coastal beaches in February and March.

Behavioral observations and sampling

On 26 November 1999, we marked 18 randomly chosen female iguanas with temporary paint numbers on their flanks for identification. These numbers do not affect the behavior of the animals (Partecke et al., 2002; Wikelski and Audet, unpublished data). Animals were watched continuously from 7:30 h to 17:30 h during the mating period, and we observed 8 of 18 (44%) females copulate (see Methods in Rubenstein and Wikelski, 2003). Marked marine iguanas were captured by hand within 30 s of approach three times during the mating period from 26 November 1999 to 9 January 2000. Females were captured once before copulations began (pre-mating), once immediately after we observed the first copulations in the population (first copulation), and once as the mating period was ending and copulations were winding down (last copulation). Animals were captured and bled during the pre-mating stage on one evening from 20:00 h to 24:00 h, during the first copulation stage on four successive evenings from 16:00 h to 22:00 h, and during the last copulation stage over 2 days from 9:00 h to 17:00 h. We focused our capture efforts during the evening because that is when marine iguanas are sleeping in rock crevices and when we can capture large numbers of marked individuals without disturbing other marked members of the population.

Unmarked female marine iguanas were captured at four different behavioral stages during the nesting period: (1) as they made their way to the nesting beach (arrival); (2) while digging nests before egg-laying (nest digging); (3) while covering nests after egg-laying (nest covering); and (4) after returning to the coastline after nesting (departure). Fifteen unique females were collected during each of the four stages. After females were sampled, they were marked with temporary paint numbers and released so that we did not duplicate captures in successive stages. Females were captured during the arrival stage on 1 day from 9:00 h to 13:00 h, during the nest digging stage on 1 day from 10:00 h to 13:00 h, during the nest covering stage over two successive days from 9:00 h to 11:00 h, and during the departure stage on two successive days from 13:00 h to 17:00 h. We focused our capture efforts for the arrival, nest digging, and nest covering stages during the midday because that is when marine iguanas were most active. We focused our efforts for the departure stage during the

afternoon because that is when it was easiest to find individuals along the coast. Individuals arriving at the nesting area were easy to identify because they spent a few days in the nearby rocks before they attempted to build a nest. Individuals digging nests faced the hole and used their front legs to excavate dirt, while individuals covering nests faced away from the hole and used their hind legs to push dirt. Females that had just departed the nesting area and arrived back at the coast were identified by their gaunt-looking bodies, which were covered in dirt and dust from the nesting beach.

Corticosterone has a circadian and a circatidal rhythm in marine iguanas, but those rhythms seem to vary with the time of year (Woodley et al., 2003). In planning our sampling regime, we tried to account for behavioral stage, capture time, and tidal regime whenever possible, but due to logistical issues, we needed to sample iguanas at different times during different stages. During the breeding season, baseline CORT in females does not vary with the tidal cycle (Woodley et al., 2003), so we did not account for tidal cycle. Baseline CORT varies with time of day, but it is only significantly elevated around midday (Woodley et al., 2003). Only 4 of 49 (8%) mating period samples were collected at midday (between 10:00 h and 14:00 h) and removing these individuals from our analyses does not change the results. The majority of our nesting samples were collected around mid-day; only the departure stage samples were collected in late afternoon. Comparing baseline CORT values between periods could thus be problematic, but given the large differences we observed, any effect of circadian (or circatidal) rhythm is not likely to be driving the between period patterns. Finally, although no one has examined the effect of circadian and circatidal rhythms on T, E₂, and P₄ in marine iguanas, we saw no effect of these rhythms on the patterns in our data.

We also collected 12 unique females that were fighting with other females (fighting females) and compared them to females in the same nesting stage (nest digging and covering stages) that were not fighting (non-fighting females). Although aggressive interactions over nest sites can take many forms (see Carpenter, 1966), we defined fighting as two females either head-butting or interlocked and grappling for at least 30 s. Most female fighting bouts tended to last less than 5 min, but the actual fight is the culmination of an aggressive interaction that begins with threats and head-bob displays between individuals that can also last for a few minutes. One of the two females in the fight was randomly captured and bled. We did not determine whether the captured female was the territory owner or intruder, nor did we determine whether the territory owner had been digging or covering a nest prior to the fight. There were no differences in body size between the fighting and the non-fighting females (mass: $t_{40} = 0.068$, $P = 0.95$; snout to vent length: $t_{40} = 0.57$, $P = 0.57$).

Blood was collected in 2 ml heparinized Vacutainer tubes (Becton Dickinson) from the caudal vein on the underside of

the tail within 3 min from the time of capture (corticosterone does not tend to rise until 3 min after capture initiation; Romero, 2004; Romero and Wikelski, 2001; Sapolsky et al., 2000) and centrifuged within 4 h of capture. Plasma was separated from red blood cells using a syringe (Hamilton Company) and mixed with 1 ml absolute ethanol in 2 ml tubes. The ethanol preservation method had been validated previously on marine iguana samples, revealing results indistinguishable from frozen samples when stored in this way for up to 4 months (Wikelski et al., 2005). After bleeding, all animals were weighed, measured for snout to vent length (SVL), sexed (*sensu* Wikelski and Trillmich, 1997), and released within 5 min of capture. At the time of plasma separation, we measured the hematocrit as the ratio of red blood cells to whole blood. Body condition was then estimated using both hematocrit and a body condition index score ($\text{mass} \times 10^6/\text{SVL}^3$) used commonly with marine iguanas (Laurie, 1989; Romero and Wikelski, 2001; Wikelski and Trillmich, 1997). Plasma samples were stored in the shade for up to 4 months and then transported with permission from the Galápagos National Park Service to Princeton University, Princeton, NJ, and kept at -20°C . All animal use and care protocols were approved by the Institutional Animal Care and Use Committees at the University of Illinois at Urbana-Champaign and Princeton University.

Hormone assays

Plasma levels of T, E₂, CORT, and P₄ were determined by four separate direct radio-immuno assays (RIAs). However, first we confirmed—via separation of hormones on a chromatography column (e.g., Wingfield and Farner, 1975)—that the main androgen hormone in female marine iguana samples was T, and not dihydrotestosterone (DHT). We analyzed 14 samples from different females from varying times during the mating and nesting periods and found that DHT and T were correlated, but DHT was always about 10 times lower than T (DHT [ng/ml] = $0.09 \times \text{T [ng/ml]}$; $r^2 = 0.56$, $P < 0.05$). We used 100 μl plasma for the determination of each hormone and conducted three or four RIAs for each hormone. Aliquots of 100 μl plasma were equilibrated with 2000 cpm of 3H-hormone overnight at 4°C , extracted with dichloromethane, dried in a 40°C water bath under nitrogen gas, and then re-dissolved in 550 μl of buffer. Samples were then allowed to equilibrate with buffer overnight at 4°C . Fractions of 200 μl were taken for duplicates used in the RIA as described previously (e.g., Wingfield and Farner, 1975; T antibody: T3003, and P₄ antibody: P-1604, both Wien Laboratories, Succasunna NJ 07876; E₂ antibody: #1702, Biogenesis, Brentwood NH 03833; CORT antibody, B3-163 Endocrine Sciences, Calabasas CA 91301). Fractions of 100 μl were directly counted for the determination of recovery (mean recoveries were 68% for T, 63% for E₂, 80% for CORT, and 73% for P₄). Two 400 μl aliquots of distilled water (water blanks)

and a total of four 400 μl aliquots containing either 0.15, 0.25, or 0.50 ng of non-radioactive hormone standards were taken through the whole assay procedure to estimate non-specific interference, assay accuracy, and intra- and inter-assay variation. Blanks were below the detection limits, which were 0.08 ng/ml for T, 0.12 ng/ml for E₂, 0.15 ng/ml for CORT, and 0.8 ng/ml for P₄. The intra-assay variations were 7%, 11%, 14% for T, 16%, 20%, 17% for E₂, 6%, 12%, 10% for CORT, and 7%, 10%, 12%, 14% for P₄. The inter-assay variations were 20% for T, 6% for E₂, 10% for CORT, and 13% for P₄.

Statistical analyses

We assessed normality of the data visually and transformed those data that deviated from normality as necessary. In cases where we were unable to transform the data, non-parametric tests were performed. All statistical analyses were conducted using JMP 5.1.2 (SAS Institute Inc., Cary, NC). We compared hormone levels between the mating and nesting periods using non-parametric Mann–Whitney tests, and condition measures between the mating and nesting periods using *t* tests. We analyzed mating period hormone and condition data using general linear models with “individual” as a random effect to account for repeated sampling of the same individuals. Nesting period hormone and condition data were analyzed using one-way analysis of variance models. When we were unable to transform data that deviated from normality, we used non-parametric Wilcoxon tests to examine hormones patterns in different stages. Post hoc analyses were conducted using Tukey HSD tests for parametric tests and multiple comparison tests for non-parametric tests. We correlated the four steroid hormones with the two condition measures for both the mating and nesting periods. For the mating period data, “individual” was included in the models as a random effect because we had repeated samples from the same females. For each of the four sets of four correlations, we used sequential Bonferroni corrections to adjust the critical values (Rice, 1989).

For the eight females where we had observed the copulation date, we used general linear models to correlate the four steroid hormones and the two conditions measures with the date the animal was sampled relative to its copulation date. “Individual” was included in the models as a random effect to account for repeated sampling of the same eight females. For P₄, we also included relative copulation date (squared) in the model because a power function fit the data better than a linear function. The relative copulation date was standardized such that the date of copulation was day 0 and capture dates before that had negative values, while capture dates after that had positive values. We used sequential Bonferroni corrections to adjust the critical values.

We used *t* tests to compare hormone levels and condition measures between fighting and non-fighting females. When we were unable to transform data that deviated from

normality, we used non-parametric Mann–Whitney tests to compare fighting and non-fighting females.

Results

Breeding patterns

Testosterone was higher during the mating period than during the nesting period ($U = 55.09$, $df = 1$, $P < 0.0001$; Fig. 1A). Testosterone increased towards the end of the mating period during the last copulation stage ($F_{2,26} = 4.18$, $P = 0.027$; Fig. 1A), while during the nesting period, it increased during nest digging and covering ($F_{3,56} = 9.02$, $P < 0.0001$; Fig. 1A). Estradiol was higher during the mating period than during the nesting period ($U = 87.03$, $df = 1$, $P < 0.0001$; Fig. 1B). It increased from the pre-mating stage to the first copulation stage and remained elevated throughout the mating period ($F_{2,26} = 36.94$, $P < 0.0001$; Fig. 1B). During the nesting period, however, E₂ was extremely low during all stages ($\chi^2 = 2.93$, $df = 3$, $P = 0.40$; Fig. 1B). During the nesting period, only 6 of 45 (13%) females had E₂ levels above the detection limit (0.12 ng/ml), and all but one had levels lower than 0.2 ng/ml. In contrast, during the mating period, all females had levels higher than 0.45 ng/ml (Fig.

1B). Corticosterone was lower during the mating period than during the nesting period ($U = 55.57$, $df = 1$, $P < 0.0001$; Fig. 1C). Corticosterone began to increase at the end of the mating period ($F_{2,29} = 26.06$, $P < 0.0001$; Fig. 1C). It was most elevated during the nesting period before eggs were laid, but declined significantly immediately after egg-laying ($F_{3,56} = 26.80$, $P < 0.0001$; Fig. 1C). Progesterone levels were similar during the mating and nesting periods ($U = 0.67$, $df = 1$, $P = 0.41$; Fig. 1D). Progesterone was elevated at the beginning of the mating period, but decreased towards the end ($F_{2,29} = 3.50$, $P = 0.044$; Fig. 1D). Moreover, progesterone increased towards the beginning of the nesting period, but then declined steadily throughout ($F_{3,56} = 24.95$, $P < 0.0001$; Fig. 1D). Hematocrit did not differ between the mating and nesting periods ($t_{106} = 0.14$, $P = 0.89$; Fig. 2A). It decreased from the pre-mating stage to the two copulation stages ($F_{2,28} = 16.22$, $P < 0.0001$; Fig. 2A). During the nesting period, hematocrit was highest during the nest digging and covering stages and lowest at the start and end of nesting ($F_{3,56} = 14.97$, $P < 0.0001$; Fig. 2A). The body condition index was higher during the mating period than during the nesting period ($t_{107} = 9.54$, $P < 0.0001$; Fig. 2A) and decreased over the course of both the mating ($F_{2,29} = 12.26$, $P = 0.0001$; Fig. 2B) and nesting periods ($F_{3,56} = 60.92$, $P < 0.0001$; Fig. 2B).

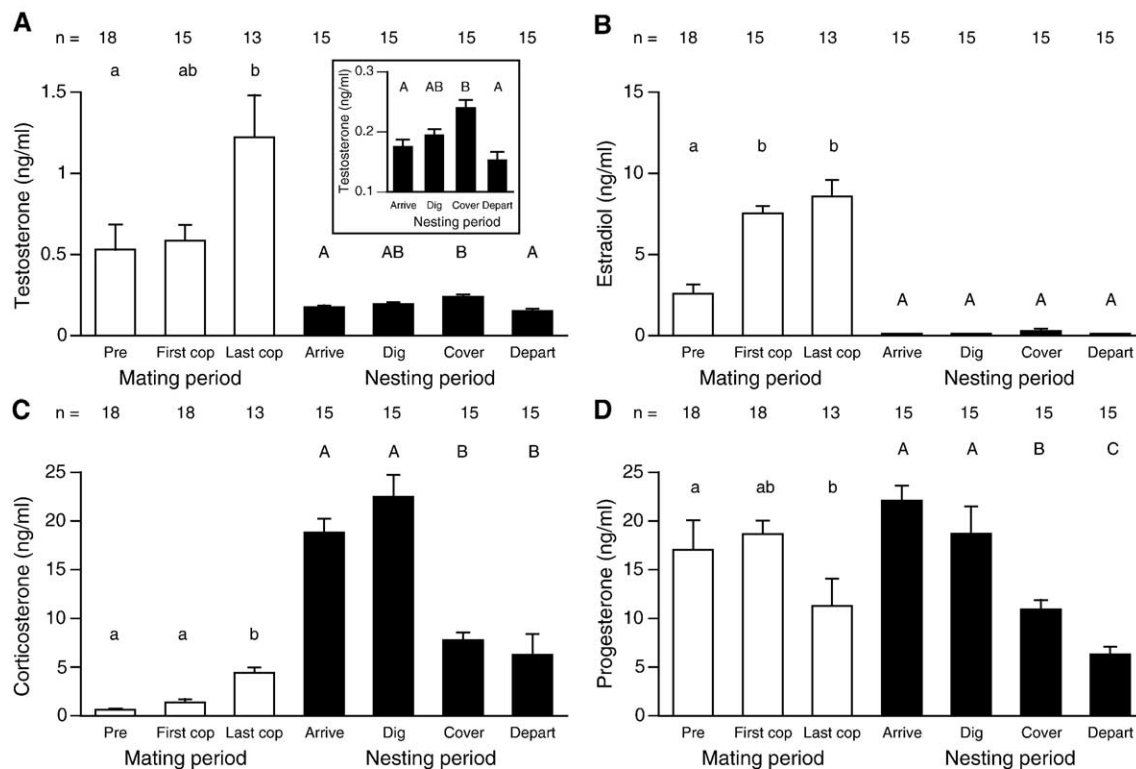


Fig. 1. Patterns of (A) testosterone, (B) estradiol, (C) corticosterone, and (D) progesterone in female Galápagos marine iguanas during the mating period (black bars) and nesting period (white bars). The mating period is divided into the pre-mating (pre), first copulation (first cop), and last copulation (last cop) stages, while the nesting period is divided into the arrival (arrive), nest digging (dig), nest covering (cover), and departure (depart) stages. Although mating and nesting period data were both shown on the same scale to accentuate the differences between the periods, testosterone patterns during the nesting period are shown on a different scale to highlight the differences among nesting stages more clearly. Lowercase letters indicate which stages were significantly different during the mating period, while capital letters indicate which stages were significantly different during the nesting period (all at $P < 0.05$).

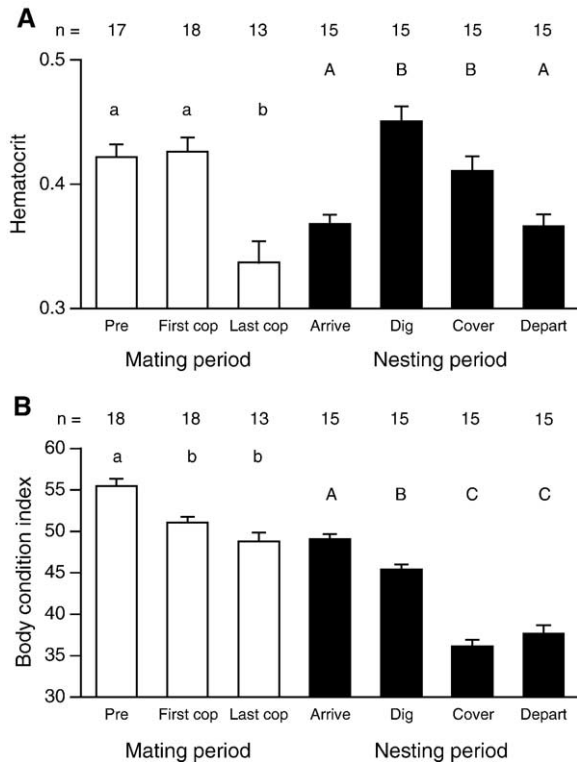


Fig. 2. Patterns of (A) hematocrit and (B) the body condition index (mass $\times 10^6/SVL^3$) in female Galápagos marine iguanas during the mating period (black bars) and nesting period (white bars). The mating period is divided into the pre-mating (pre), first copulation (first cop), and last copulation (last cop) stages, while the nesting period is divided into the arrival (arrive), nest digging (dig), nest covering (cover), and departure (depart) stages. Lowercase letters indicate which stages were significantly different during the mating period, while capital letters indicate which stages were significantly different during the nesting period (all at $P < 0.05$). Note that the drop in body condition from nest digging to covering is largely caused by the mass loss due to egg-laying.

There was a significant positive correlation between hematocrit and the body condition index during the mating period ($F_{1,29} = 7.20, P = 0.011, r = 0.66$), but not during the

nesting period ($F_{1,58} = 0.34, P = 0.56, r = 0.24$). Testosterone was negatively correlated with both hematocrit and the body condition index, while E_2 was only negatively correlated with the body condition index during the mating period (Table 1). Corticosterone was negatively correlated with hematocrit (but not the body condition index) during the mating period, but it was positively correlated with the body condition index during the nesting period (Table 1). Finally, P_4 was slightly positively correlated with the body condition index during the nesting period (Table 1).

For the eight females that copulated, there were significant positive relationships between the time before/after copulation and T ($F_{1,14} = 8.93, r^2 = 0.43, P = 0.0098$; Fig. 3A), E_2 ($F_{1,14} = 15.97, r^2 = 0.59, P = 0.0013$; Fig. 3B), and CORT ($F_{1,15} = 19.83, r^2 = 0.60, P = 0.0005$; Fig. 3C). Progesterone, however, increased up until the day of copulation, but decreased afterwards (time before/after copulation: $F_{1,13} = 3.63, P = 0.079$; time before/after copulation (squared): $F_{1,13} = 11.59, P = 0.0047; r^2 = 0.70$; Fig. 3D). There was also a significant negative relationship between the time before/after copulation and hematocrit ($F_{1,15} = 17.45, P = 0.0008$; Fig. 4A) and the body condition index ($F_{1,15} = 8.51, P = 0.011$; Fig. 4B).

Female aggression

During the mating period, T and E_2 were significantly higher during the last copulation stage (when female–male aggression is most common) than during the pre-mating stage, but not higher than during the first copulation stage (Figs. 1A and B). Corticosterone was significantly higher during the last copulation stage than during both of the other stages (Fig. 1C), while P_4 was only significantly lower during the last copulation stage than during the pre-mating stage (Fig. 1D). In those females that we observed copulating, however, E_2 (Fig. 3B) and CORT (Fig. 3C) tended to increase steadily up to and beyond the day of

Table 1

Correlations between condition measures (hematocrit, body condition index) and steroid hormones (testosterone, estradiol, corticosterone, progesterone) during the mating and nesting periods

Hormone	Mating period		Nesting period	
	Hematocrit	Body condition index	Hematocrit	Body condition index
Testosterone	$F_{1,26} = 11.78$ $r = -0.74$ $P = 0.002^*$	$F_{1,27} = 13.89$ $r = -0.75$ $P = 0.0009^*$	$F_{1,58} = 2.66$ $r = 0.21$ $P = 0.11$	$F_{1,58} = 3.76$ $r = -0.25$ $P = 0.058$
Estradiol	$F_{1,26} = 1.66$ $r = -0.59$ $P = 0.21$	$F_{1,27} = 8.13$ $r = -0.68$ $P = 0.0082^*$	$F_{1,58} = 0.91$ $r = -0.12$ $P = 0.34$	$F_{1,58} = 1.61$ $r = -0.16$ $P = 0.21$
Corticosterone	$F_{1,29} = 13.97$ $r = -0.68$ $P = 0.0008^*$	$F_{1,30} = 3.31$ $r = -0.54$ $P = 0.079$	$F_{1,58} = 4.78$ $r = 0.28$ $P = 0.033$	$F_{1,58} = 60.74$ $r = 0.72$ $P < 0.0001^*$
Progesterone	$F_{1,29} = 2.36$ $r = 0.62$ $P = 0.14$	$F_{1,30} = 2.91$ $r = 0.62$ $P = 0.72$	$F_{1,58} = 0.37$ $r = 0.08$ $P = 0.54$	$F_{1,58} = 28.93$ $r = 0.58$ $P < 0.0001^*$

For the mating period data, “individual” was included in the models as a random effect because we had repeated samples from the same females. Data were sequentially Bonferroni corrected for each of the four dependent variables (hormones).

* Indicates significance after a sequential Bonferroni correction.

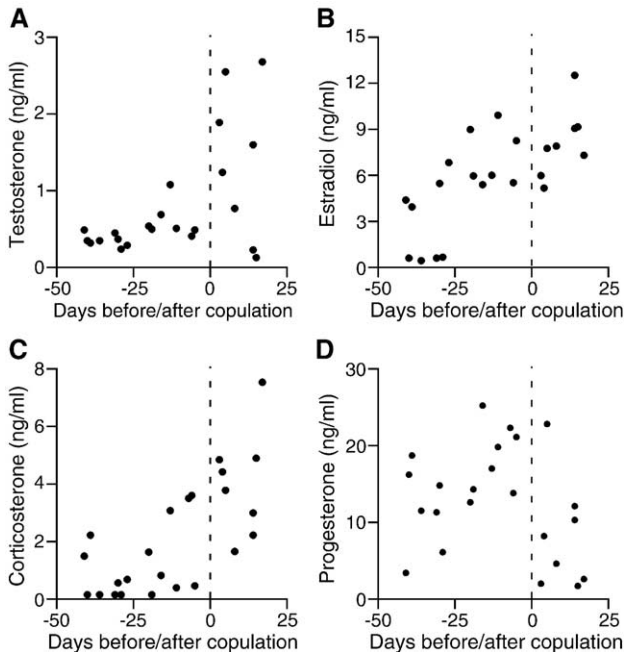


Fig. 3. Patterns of (A) testosterone, (B) estradiol, (C) corticosterone, and (D) progesterone in female Galápagos marine iguanas captured during the mating period. Data points are not independent; eight females were each recaptured three times over the course of the mating period (45 days). One female is missing testosterone and estradiol data from one of the capture periods, while another female is missing progesterone data from one of the periods. Points are standardized to the day of copulation, which is indicated by the dotted line.

copulation, while T tended to increase sharply (Fig. 3A), and P_4 tended to decrease sharply, immediately after copulating (Fig. 3D). During the nesting period, T was significantly higher during the nest covering stage than during the arrival and departure stages, but not higher than during the nest digging stage (Fig. 1A). Estradiol was equally low at all nesting stages (Fig. 1B). Corticosterone and P_4 were significantly lower during the nest covering stage than during the arrival and nest digging stages (Figs. 1C and D).

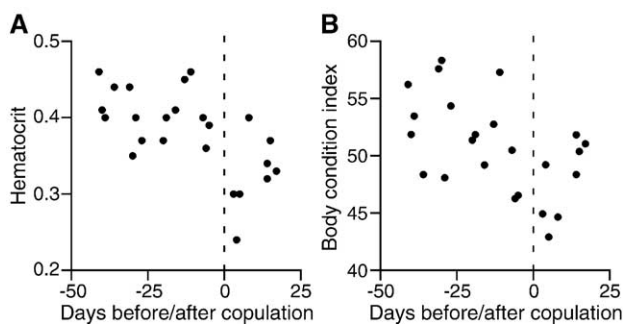


Fig. 4. Patterns of (A) hematocrit and (B) the body condition index (mass $\times 10^6/\text{SVL}^3$) in female Galápagos marine iguanas captured during the mating period. Data points are not independent; eight females were each recaptured three times over the course of the mating period (45 days). Points are standardized to the day of copulation, which is indicated by the dotted line.

Testosterone was significantly lower in fighting females than in non-fighting females ($t_{40} = 9.01$, $P < 0.0001$; Fig. 5A), but E_2 was significantly higher in fighting females than in non-fighting females ($U = 27.73$, $df = 1$, $P < 0.0001$; Fig. 5B). A single non-fighting female had an E_2 level more than four standard deviations above the mean. If we remove this individual from the analysis, the mean E_2 level in non-fighting females drops by nearly half from 0.20 ng/ml to 0.126 ng/ml and the trend becomes even more significant ($U = 32.15$, $df = 1$, $P < 0.0001$). Corticosterone did not differ between the two classes ($t_{40} = 0.065$, $P = 0.95$; Fig. 5C), but P_4 was significantly higher in fighting females than in non-fighting females ($t_{40} = 4.68$, $P < 0.0001$; Fig. 5D). Hematocrit was slightly lower in fighting females than in non-fighting females ($t_{40} = 2.30$, $P < 0.027$; Fig. 6A), but the body condition index did not differ between the two classes ($t_{40} = 0.045$, $P = 0.66$; Fig. 6B).

Discussion

Steroid patterns during mating and nesting

Plasma steroid concentrations in female Galápagos marine iguanas changed predictably during the mating and nesting periods and the patterns resembled those of other viviparous lizards (Amey and Whittier, 2000; Jones et al., 1997; Radder et al., 2001; Rhen et al., 2003; Shanbhag et al., 2001). Testosterone reached maximal levels during the mating period (most likely during vitellogenesis; Fig. 1A) and were much lower during nesting. The role of T in

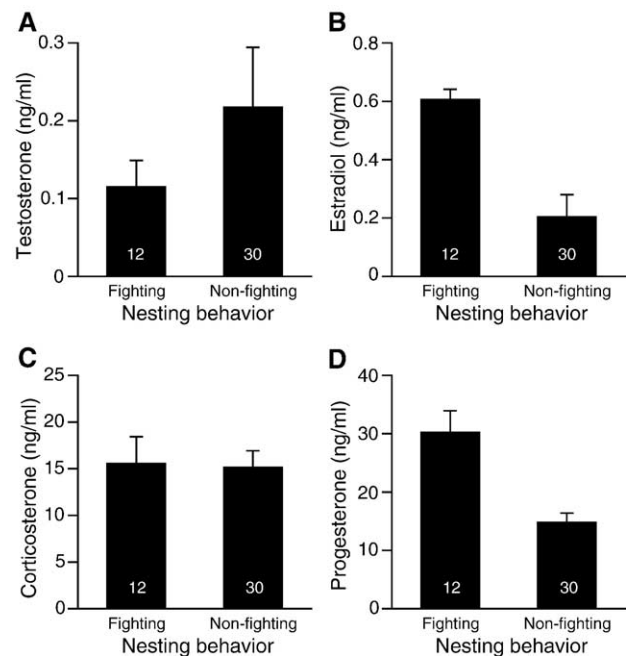


Fig. 5. Levels of (A) testosterone, (B) estradiol, (C) corticosterone, and (D) progesterone in female Galápagos marine iguanas captured while fighting or not fighting (i.e., digging or covering nests) during the nesting period.

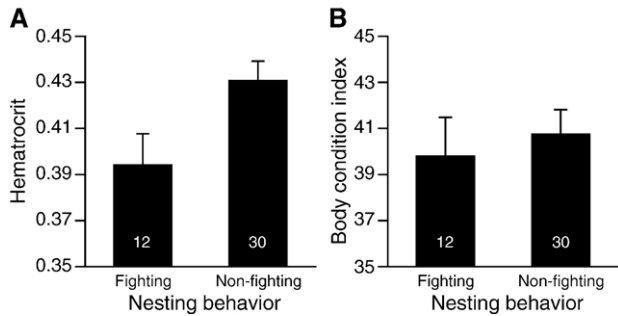


Fig. 6. Levels of (A) hematocrit and (B) the body condition index (mass $\times 10^6/\text{SVL}^3$) in female Galápagos marine iguanas captured while fighting and not fighting (i.e., digging or covering nests) during the nesting period.

females during breeding is poorly understood (Staub and DeBeer, 1997), but in many reptiles, T tends to increase during mating and vitellogenesis (Cree et al., 1992; Edwards and Jones, 2001; Guillette et al., 1997; Hamann et al., 2002; Ott et al., 2000; Rhen and Crews, 2000; Saint-Girons et al., 1993; Taylor et al., 2004; Whittier et al., 1987) and may stimulate oviductal hypertrophy (Jones and Guillette, 1982) and uterine development (Yaron, 1972b). Alternatively, this increase could occur because T is simply a precursor to E_2 (Bentley, 1998), it may interact directly with E_2 in regulating vitellogenesis (Callard et al., 1990), or it may be related to mating behaviors as it is in males (Lindzey and Crews, 1986). We also found a negative relationship between T and body condition during the mating period, which may be related to the high costs of mate choice in female marine iguanas (Wikelski et al., 2001).

Estradiol peaked during the mating period, but was extremely low during the nesting period (Fig. 1B). During breeding, E_2 is generally responsible for vitellogenesis (Edwards and Jones, 2001; Guillette et al., 1997; Ho et al., 1982; Ott et al., 2000; Radder et al., 2001; Rhen et al., 2003; Taylor et al., 2004). Moreover, E_2 (either alone or with P_4) can stimulate attractivity or receptivity of female reptiles (Mason and Adkins, 1976; McNicol and Crews, 1979; Rhen and Crews, 2000; Tokarz and Crews, 1980; Whittier and Tokarz, 1992; Winkler and Wade, 1998). Estradiol levels in female lizards tend to be extremely low during gestation, parturition, and post-parturition (Taylor et al., 2004).

Corticosterone rose throughout the mating period and remained elevated during nesting until levels dropped off sharply immediately after egg-laying (Fig. 1C). Corticosterone tends to increase during breeding in most taxa, including reptiles (Romero, 2002), which may be related to energy mobilization for vitellogenesis (Grassman and Crews, 1989; Wilson and Wingfield, 1994). Many lizards also show peaks in CORT during the late stages of gestation (Dauphin-Villemant et al., 1990; Guillette et al., 1997), which may be related to physiological changes leading up to parturition (Taylor et al., 2004). In marine iguanas, body condition tended to decrease during both the mating and nesting periods, while CORT tended to increase.

Progesterone decreased towards the end of the mating period, but then rose at the beginning of the nesting period, only to decrease continually throughout nesting (Fig. 1D). During breeding, P_4 is related to the maintenance of oviduct vascularity (Mead et al., 1981; Yaron, 1972a), as well as inhibition of follicular growth and oviductal contractility (Guillette et al., 1991). In many taxa, P_4 tends to increase at the beginning of the breeding season, and then decrease slightly towards the end (Nelson, 2000). During nesting, P_4 tends to peak at the beginning of gestation, but decline as parturition approaches (Bonnet et al., 2001; Taylor et al., 2004). These patterns suggest that egg-laying may actually be stimulated by a drop (Taylor et al., 2004), and inhibited by an increase, in P_4 (Guillette et al., 1991; Shanbhag et al., 2001). Overall, our results suggest that reproductive events (e.g., ovulation, vitellogenesis, and egg-laying) during the breeding season are regulated by multiple steroid hormones in marine iguanas and mimic patterns seen in other reptiles (Edwards and Jones, 2001).

Female aggression and the challenge hypothesis

Since steroid hormones in females are generally elevated during the early breeding season and are directly related to many physiological phases of pregnancy, it is difficult to correlate steroid levels and periods of increased aggression around mating. Nonetheless, we predicted that steroid hormones related to aggressive behavior during the mating period would be elevated during the stage after copulations occurred. Testosterone, E_2 , and CORT tended to increase during the last copulation stage, but only T increased sharply immediately after mating in those females we observed copulating. Moreover, P_4 declined in the last copulation stage and decreased sharply immediately after copulating. These results suggest that an increase in T could be related to female–male aggression during the mating period. Moreover, given the dramatic change in body color during the last copulation stage, these changes in hormone levels could also be directly related to these post-copulatory morphological changes.

To better understand the neuroendocrine correlates of female aggression and to disentangle the effects of elevated steroids during the mating period, we examined a variety of potentially important steroids related to female–female aggression during the nesting period when baseline levels of T and E_2 were relatively low. We predicted that steroid hormones related to aggressive behavior during the nesting period would be elevated during the nest digging and covering stages when female aggression was most intense. We found that T was, in general, much lower than during the mating period, but significantly elevated during the nest covering stage. Estradiol was extremely low during all nesting stages. High but declining patterns of baseline CORT and P_4 throughout nesting suggest that these hormones might be more related to egg-laying than to aggression (*sensu* Taylor et al., 2004). Thus, since only T

was elevated during the nesting stages when aggression was most common, our results suggest that, like during the mating period, T may be related—either directly or indirectly—to female–female aggression in marine iguanas as it is in other reptiles (Rhen et al., 1999). Similarly, in colonial cliff swallows, T in females peaks during the nesting stages when intrasexual aggression is highest (Smith et al., 2005).

Despite an increase in T during the nest covering stage when females tend to be most aggressive, baseline levels in non-aggressive situations do not necessarily relate directly to aggressive behavior (Ramenofsky, 1984). The challenge hypothesis only makes the prediction that T should be high during aggressive encounters, or times of social instability (Wingfield et al., 1990). To test this prediction, we compared steroid levels in females caught during fights over nest sites to non-fighting females captured during similar reproductive stages. Because we found elevated E_2 and P_4 , but reduced T, in fighting compared to non-fighting females, we speculate that E_2 and P_4 may be directly, while T may be indirectly, related to female–female aggression (*sensu* Rhen et al., 1999). Our data are consistent with the hypothesis that fighting females rapidly aromatize T into E_2 at a time when baseline E_2 levels are barely detectable, but T levels are high (Fig. 7). Such a hypothesis is based upon the knowledge that in mammals, T often has no direct effect on female aggression (Payne and Swanson, 1972; Takahashi and Lisk, 1983), but in many female lizards E_2 can directly modulate aggressive behavior (Rhen et al., 1999; Woodley et al., 2000a; Woodley and Moore, 1999b). In mammals (Dessi-Fulgheri et al., 1976) and in birds (Silverin et al., 2004), aromatase activity in the brain is correlated with

aggression levels in males, and we would expect a similar pattern in females. Aromatization could occur at various tissues including the ovaries (Bobes et al., 2003; Gómez et al., 1998; Wasson and Watts, 2000), spine (Blomqvist, 2000; Evrard and Balthazart, 2003, 2004; Evrard et al., 2000; Freking et al., 2000), and/or brain (Balthazart and Ball, 1998; Schlinger and Arnold, 1991, 1992; Schlinger and Arnold, 1993). Thus, this hypothesis, namely that T is being rapidly aromatized to E_2 during aggressive encounters (either as a cause or as a consequence of aggressive behavior), explains both why we observed elevated E_2 , but reduced T, in fighting females, as well as why we saw elevated baseline levels of T and extremely low baseline levels of E_2 in non-fighting females during the nest covering stage when aggressive interactions are most common.

Although elevated P_4 may be related to female–female aggression, it is not clear whether it is related directly or indirectly. There is increasing evidence that P_4 , like E_2 and other steroids, can have rapid non-genomic effects on behavior (Cross and Roselli, 1999; Frye, 2001; Moore and Evans, 1999; Remage-Healey and Bass, 2004), leading to an increase in aggression in some species (Davis and Marler, 2003; Kapusta, 1998; Weiss and Moore, 2004), but a decrease in others (reviewed in Davis and Marler, 2003). Alternatively, P_4 could be indirectly metabolized to E_2 via androstenedione (and possibly T) during aggressive interactions (Bentley, 1998, Fig. 7).

Although we found changes in T, E_2 , and P_4 that were related to aggressive behavior, it is unclear whether the changes in hormone levels cause a change in behavior or are an immediate consequence of a change in behavior (Yang and Wilczynski, 2002). Although fights only lasted for a

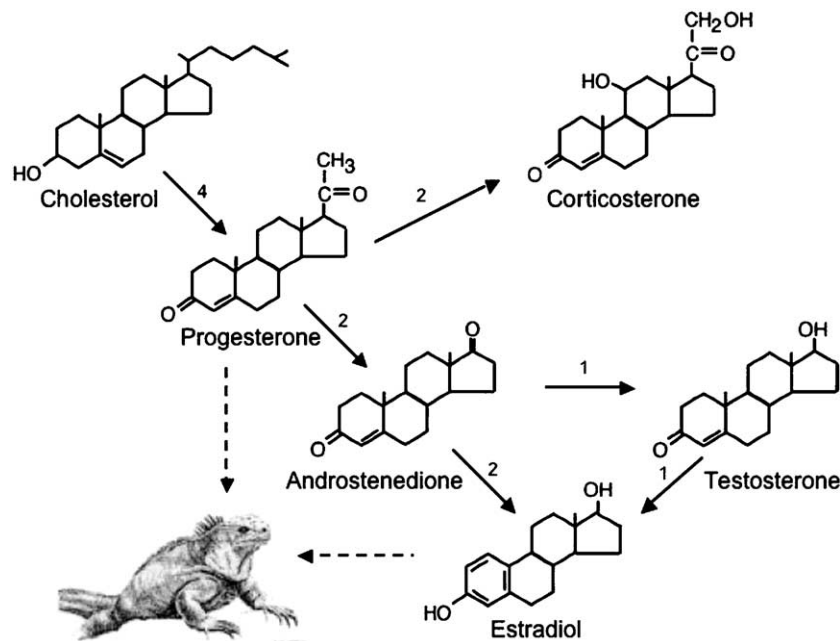


Fig. 7. Interrelationships among testosterone, estradiol, corticosterone, and progesterone (modified from Bentley, 1998). Solid arrows represent simplified metabolic pathways with the numbers above each arrow indicating the number of enzymatic steps between the two metabolites. Dotted arrows represent hypothesized direct, rapid hormone–behavior interactions among steroids and female aggression in Galápagos marine iguanas.

matter of minutes, aggressive interactions always commenced a few minutes before they escalated to fights with a series of threats and head-bob displays. This lengthy time course (ca. 1–10 min) of aggressive interactions would allow sufficient time for either rapid changes in steroid hormones that could alter behavior, or alternatively, changes in hormone levels in response to changes in behavior. Nonetheless, female marine iguanas appear to show a rapid and facultative response during aggressive encounters, producing increased E_2 and P_4 —to levels that are higher than at any other stage during nesting—only during fights. Since female body condition is decreasing throughout nesting, elevated E_2 and P_4 may have energetic costs if these steroids cause them to behave aggressively more often, leading to a further reduction in body condition. Moreover, elevated P_4 could also be costly because it could inhibit egg-laying (Guillette et al., 1991; Shanbhag et al., 2001).

Our results support the challenge hypothesis in female marine iguanas and suggest that T, E_2 , and P_4 may all be related to female aggression. Although baseline levels of T were elevated and baseline levels of E_2 were extremely low during the nesting stages when female–female aggression was most common, E_2 and P_4 were higher, and T was lower, in fighting versus non-fighting females. These results suggest that E_2 and P_4 may be directly related, while T may be only indirectly related, to female–female aggression in a maternal context. Similarly, during the mating period, baseline levels of T increased, while P_4 decreased, sharply after copulating, a time when females become aggressive towards males. Although these results suggest that T may be related to female–male aggression in a reproductive context, we might expect a similar pattern of steroid metabolism of T into E_2 if we were to examine steroid levels during aggressive interactions like we did during the nesting period.

Overall, these results suggest a complicated pathway of steroid metabolism—in which a variety of steroids and their metabolites could be working independently or in concert to alter or respond to female behavior (Davis and Marler, 2003; Woodley and Moore, 1999a)—that can be activated facultatively and rapidly during encounters (Fig. 7). It will be important to determine whether changes in hormone levels are causal or responsive to changes in behavior (Yang and Wilczynski, 2002). Moreover, further studies are needed to untangle the various possibilities for interactions and precursors such as dehydroepiandrosterone (DHEA) that may also affect aggression (Hau et al., 2000, 2004), and if these hormones may be related to different types of female aggression in different social and reproductive contexts. Future work could also measure enzymatic activity of the various enzymes involved in the production and metabolism of these steroids in the ovaries and brain (Edwards et al., 2005), and implant studies with exogenous hormone and hormone blockers will help to determine which steroids are working directly and which are working indirectly (Wood-

ley and Moore, 1999b; Woodley et al., 2000a). Many other species of iguanas exhibit high levels of female–female aggression and nest defense (for up to a month) when nesting sites are limited (reviewed in Iverson et al., 2004; Wiewandt, 1982), and would also make excellent model systems. Our data suggest that the rapid production and conversion of steroid hormones may be an important mechanism underlying female aggression in reptiles and other vertebrates and that these topics deserve further study.

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