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Developmental Effects of Testosterone on Behavior in Male and Female Green Anoles (*Anolis carolinensis*)

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This study addressed the role of testosterone (T) in the development of sexually dimorphic behavior in the green anole lizard, *Anolis carolinensis.* We documented the pattern of endogenous T concentrations during ontogeny and we determined the behavioral effects of experimentally elevated T in juvenile males and females. T concentrations were measured in the plasma of hatchlings from eggs incubated in the laboratory, in juveniles of all sizes sampled in the field, and in the yolks of freshly laid eggs in the laboratory and were compared to plasma T in adult females (measured in this study) and adult males. There were no sex differences in plasma T in hatchling and small juvenile (<26-mm snout–vent length, SVL; <14 days old) males and females, concentrations of which in both sexes tended to decline over the 14-day posthatching period. Plasma T sharply increased in juvenile males, but not females, after approximately 14 days posthatching (>25-mm SVL), and it became significantly higher after approximately 38 days posthatching (>30-mm SVL). Plasma T for juvenile males was within the range detected in breeding adult females, but it was 20- to 45-fold lower than that of adult males, breeding or postbreeding. All eggs contained detectable yolk T, but eggs that gave rise to males contained nearly twice as much yolk T as those that gave rise to females. We do not know whether this yolk T comes from the mother, embryo, or both. In behavior trials conducted in the laboratory, juveniles (36- to 42-mm SVL) with T implants, regardless of whether they were male or female, had increased activity levels compared to juveniles with blank implants, due to increased rates of nearly every behavior monitored. These results are discussed in the context of the organization–activation theory of sexual differentiation and the particular life history of *A. carolinensis.*

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Selection pressures acting on males and females frequently result in sexual dimorphisms in morphology and behavior (Shine, 1989; Andersson, 1994). As a guiding principle, the organization–activation theory (first formulated by Phoenix, Goy, Gerall, and Young, 1959) has been useful for elucidating the nature of endocrine effects on sex differences. This theory states that steroid exposure can affect sexual dimorphism by two general mechanisms. First, during a critical period in early development, steroids can organize long-lasting sexual dimorphisms in the nervous system that result in morphological or behavioral trait expression in the organized sex. Organizational effects are typically considered irreversible, in that they persist without subsequent steroid exposure (Arnold and Breedlove, 1985; Moore, 1991; Wade, 1999). Second, steroid exposure later in life can activate (or facilitate) expression of sex differences by targeting the previously organized structures. Activational effects are transient, present only when steroid concentrations are above the threshold for response (Arnold and Breedlove, 1985; Moore, 1991; Wade, 1999). Research across vertebrate taxa supports the idea that gonadal steroids can be important regulators of sex differences (reviewed in Kelley, 1988; Ketterson and Nolan, 1992; Moore and Lindzey, 1992; Whittier and Tokarz, 1992; Cooke, Hegstrom, Villeneuve, and Breedlove, 1998; Wade, 1999). However, organization and activation do not always occur as distinctly separate processes (Arnold and Breedlove, 1985). Furthermore, in addition to the traditional view of sexual dimorphism as requiring both organization and activation, some traits may

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require only organization or only activation, or they may arise by extrasteroidal mechanisms (Cooke *et al.*, 1998; Moore, Hews, and Knapp, 1998; Wade, 1999).

Few species have been studied for both endogenous steroid concentrations during ontogeny and steroid effects on sexual dimorphism. The green anole lizard, *Anolis carolinensis*, offers an opportunity to conduct such a study within a life history framework in which sex differences in adult morphology, behavior, and physiology have been well studied. In general, the morphological sex differences associated with courtship and reproduction appear to be insensitive to androgen manipulation in adults (e.g., the neural and muscular structures regulating dewlap, or throat fan, extension; O'Bryant and Wade, 1999). However, androgens are necessary for the full expression of adult male-typical behavior in *A. carolinensis* and other anoline species. Gonadectomy or treatment with cyproterone acetate (an anti-androgen) can greatly reduce aggression, courtship, and copulatory behavior in males (Mason and Adkins, 1976; Crews, Traina, Wetzel, and Muller, 1978; Tokarz, 1995), and exogenous testosterone (T) can reinstate or maintain these behaviors (Mason and Adkins, 1976; Adkins and Schlesinger, 1979; Winkler and Wade, 1998). Seasonal changes in T and behavior found in natural populations corroborate these experimental results. Adult males have high plasma T concentrations during the breeding season when sex differences in behavior are great and low plasma T concentrations during the nonbreeding season when sex differences in behavior are minimal or nonexistent (Jenssen, Greenberg, and Hovde, 1995; Jenssen, Congdon, Fischer, Estes, Kling, Edmands, and Berna, 1996; Tokarz, McMann, Seitz, and John-Alder, 1998; Jenssen, Lovern, and Congdon, in press). Furthermore, T supplementation can masculinize adult female behavior (Mason and Adkins, 1976; Adkins and Schlesinger, 1979), although it remains unclear whether the effect is equivalent to that seen in adult males (see Winkler and Wade, 1998).

The above studies suggest that adult male-typical behavior during the breeding season is naturally activated by T in *A. carolinensis* and that T supplementation might also activate a similar, male-typical response in adult females. However, our ability to fully evaluate the likelihood that organization, activation, or both play a role in the expression of behavioral sex differences in this species is hindered for two reasons. First, although endogenous T concentrations have been measured in adult males (Jenssen *et al.*, in press), they have not been documented in juveniles and in adult females. Second, the effects of T on the ontogeny of behavioral sex differences are unknown.

In the present study, we first documented endogenous plasma T concentrations in hatchlings, juveniles, and adults. We also documented yolk T concentrations in eggs on the day of oviposition. Second, we experimentally elevated plasma T concentrations in large (36- to 42-mm snout–vent length, SVL; approximately 60–90 days old) juveniles with T implants and subsequently monitored their behavior in pairwise laboratory trials in comparison to the behavior of controls (juveniles with blank implants). These data were used to examine the likelihood that T plays a role in only organization, organization and activation, or only activation, of the expression of adult male-typical behavior in *A. carolinensis*. If only organization is required, then (1) during early ontogeny, T should be higher in males than in females, and (2) associated with this hormone difference, both blank- and T-implanted juveniles should exhibit permanent sex differences in behavior. That only organization is required seems highly unlikely given the association of T levels with behavior in adult males as described above. More likely is one of the following two possibilities. If both organization and activation by T are required for the expression of adult male-typical behavior, then (1) during early ontogeny, T should be higher in males than in females and (2) T-implanted juvenile males should show a greater behavioral response than T-implanted juvenile females to T elevation (assuming the threshold for response is met). However, if only activation is required for the expression of adult male-typical behavior, then (1) T should not differ between males and females during early ontogeny and (2) T-implanted juveniles should show a similar behavioral response regardless of sex, greater than that observed in blank-implanted juveniles.

METHODS

Study Animal

Unlike some reptiles that exhibit temperature-dependent sex determination (TSD; Bull, 1980), *A. carolinensis* has genotypic sex determination (GSD; Viets, Ewert, Talent, and Nelson, 1994). Sexually dimorphic behavior in adults emerges within a polygynous mating system in which males attempt to establish territories that exclusively overlap those of multiple females for the duration of the 4-month (April–July) breeding season (Ruby, 1984; Andrews, 1985; Jenssen

et al., 1995; Jenssen and Nunez, 1998). Adults and juveniles of both sexes share a signal repertoire of three headbobbing display types (labeled A, B, and C; DeCourcy and Jenssen, 1994; Jenssen, Orrell, and Lovern, 2000; Lovern, 2000a). However, adult male and female display use differs considerably (Jenssen *et al.*, 2000). During territory patrol, males frequently give advertisement (i.e., undirected) displays, whereas females rarely or never do (Nunez, Jenssen, and Ersland, 1997; Jenssen *et al.*, 2000). Intermale aggression is intense, involving high display rates and the use of numerous display modifiers (aggressive postures or movements added to headbobbing displays and designed to enhance apparent body size; Greenberg, 1977; Jenssen, 1977). Furthermore, these behaviors occur within a ritualized pattern of approaching, circling, jaw sparring, and ultimately jaw locking if one of the males does not retreat (Greenberg and Noble, 1944; Jenssen *et al.*, 2000). Interfemale aggression involves lower display rates, less frequent use of display modifiers, no ritualized aggression, and little or no escalated fighting (Nunez *et al.*, 1997; Jenssen *et al.*, 2000). During courtship, both males and females display, but without using the display modifiers seen in aggressive interactions (Greenberg and Noble, 1944; Jenssen and Nunez, 1998; Winkler and Wade, 1998). Males also extend their dewlaps during courtship, but females do not (Greenberg and Noble, 1944; Winkler and Wade, 1998). Receptive females, but not males, adopt a characteristic "neck-bend" posture that facilitates the male's grip during copulation (Greenberg and Noble, 1944; Jenssen and Nunez, 1998; Winkler and Wade, 1998).

Animal Collection and Maintenance

In 1998 and 1999, adult and juvenile lizards were collected by hand or noose from a well-studied population along the Augusta Canal near Augusta, Georgia (e.g., Jenssen *et al.*, 1995; Lovern, 2000b) and maintained in the laboratory singly in plywood cages measuring 30 × 60 × 60 cm. We exposed them to a 14:10 h light:dark cycle using four 40-W full-spectrum bulbs (Durotest Vita-Lite Plus) placed on the top of each cage. Temperatures inside each cage ranged from 27 to 34°C during the day and dropped to 23°C at night. All cages contained wooden dowels for perching, numerous pieces of artificial vegetation, and a dish of moist potting soil for egg-laying (when housing gravid females). We watered and fed lizards daily on vitamin-dusted crickets. Animals used in this study were collected with permission from the Georgia Department of Natural Resources and all experimental protocols were approved by the Virginia Tech Animal Care Committee.

Plasma Collection

For collecting plasma samples from hatchlings, 16 gravid females were brought into the laboratory in May of 1998. We checked for eggs daily and incubated gathered eggs individually in plastic cups containing a vermiculite:water mix (1:1 by mass). A small depression was made on the surface of the mixture and eggs were placed so that they were half-buried. The cups were then covered with plastic wrap secured by a rubber band and incubated at 24–31°C on a diel cycle. We collected plasma samples from 18 males and 18 females on the day of hatching (90% hatching success; 36 of 40 eggs). Sex was determined by noting the presence (male) or absence (female) of enlarged postanal scales, a sexual dimorphism present before hatching (Pearson and Licht, 1974). Because of the small size of hatchlings (0.2–0.3 g), blood was collected from the trunk immediately following decapitation. Plasma (4–9 μl) was isolated from whole blood following centrifugation, transferred to 1.0-ml microcentrifuge tubes, and stored at −80°C until analysis.

In the field, we collected plasma samples from 32 juvenile males and 26 juvenile females (i.e., not yet physiologically capable of reproduction; ≤42-mm SVL), as well as from 25 adult females (>45-mm SVL). On collection dates during July and August of 1998, juveniles were captured by hand, sexed, measured for SVL to the nearest millimeter, and bled from the trunk. On collection dates during April–July (breeding) and September (postbreeding), adult females were captured by noose, sexed, and measured, and blood was collected from the postorbital sinus, after which the females were released. For all lizards, the elapsed time from initial sighting to blood collection was always <10 min to minimize potential stress effects on circulating T concentrations (e.g., Moore, Thompson, and Marler, 1991). Blood samples were kept cool on ice while in the field. Within 5 h of collection, samples of 5–75 μl plasma were isolated from whole blood by centrifugation and subsequently frozen in dry ice for transportation back to the laboratory, where they were stored at −80°C until analysis. These plasma samples were analyzed simultaneously (see below) with plasma samples from 12 adult males (collected in April and September of 1998, contemporaneously with the adult females) used in Jenssen *et al.* (in press), to which plasma T values from

juveniles and adult females in the present study are compared.

The 94 juvenile lizards from which plasma samples were obtained were divided into five size classes: (1) hatchlings (n = 36); (2) <26-mm SVL (n = 11); (3) 26- to 30-mm SVL (n = 15); (4) 31- to 35-mm SVL (n = 14); and (5) 36- to 42-mm SVL (n = 18). Based on growth rates from Michaud (1990) for *A. carolinensis*, these size classes represented age classes of 0, <14, 14–37, 38–61, and 62–100 days. Class 1 lizards were sampled on the day they hatched following incubation in the laboratory, and lizards in size classes 2–5 were all sampled in the field. The 25 adult females sampled in this study had SVL measurements of 52.9 ± 0.9 mm and were at least 180 days old.

Yolk Collection

Yolk T concentrations were determined for eggs laid by eight females collected in May of 1999 and housed singly in the laboratory. Cages were checked daily for eggs, and 15–35 mg of yolk was withdrawn from each of 22 eggs on the day of oviposition through a sterile 26-gauge needle. Yolk samples were stored in 1.5-ml microcentrifuge tubes and identified by mother, date of oviposition, and amount of yolk collected to the nearest milligram (weighed on a Mettler AE 240 balance). Immediately after being weighed, yolk samples were homogenized in 0.5 ml distilled water using a vortex mixer and facilitated by the addition of two or three small glass beads and then stored at −80°C until analysis. After withdrawal of yolk samples, eggs were incubated and sexed as described above. Nineteen of the 22 eggs collected for yolk T analyses hatched (86%), of which 6 were males and 13 were females.

T Assays

Yolk and plasma concentrations of T were measured by radioimmunoassay (RIA), following extraction and chromatographic separation, as described by Wingfield and Farner (1975), Moore (1986), and Schwabl (1993). Samples were equilibrated overnight at 5°C with 1000 cpm of [^{3}H]T (NET-553, Dupont NEN) for individual recovery determinations. Additionally, five replicate aliquots from a standard of known concentration were run in each assay and treated identically to samples, to determine intra-assay precision and inter-assay repeatability. Yolk samples were extracted twice with 3 ml petroleum ether: diethyl ether (30:70 v:v), dried under a stream of nitrogen (N), and reconstituted in 1 ml of 90% ethanol. The extracted samples were stored at −20°C overnight and then centrifuged at 2000 rpm for 5 min to precipitate neutral lipids and proteins. The supernatant was dried with N and reconstituted in 300 μl of 10% ethyl acetate in isooctane. Plasma samples were extracted twice with 2 ml diethyl ether, dried with N, and reconstituted in 300 μl of 10% ethyl acetate in isooctane. To remove additional neutral lipids and to isolate T, samples were transferred to diatomaceous earth (Celite, Sigma) microcolumns for chromatographic separation. Columns consisted of a Celite: ethylene glycol:propylene glycol upper phase (6:1.5:1.5 w:v:v) and a Celite:distilled water (3:1 w:v) lower phase. Neutral lipids were eluted from the columns with 2 ml of 100% isooctane, dihydrotestosterone was eluted with 1.5 ml of 10% ethyl acetate in isooctane, and T was eluted with 2.5 ml of 20% ethyl acetate in isooctane. The purified T fractions were dried with N, resuspended in sample buffer, and then placed overnight at 5°C.

Competitive binding RIA was performed with [^{3}H]T and T antiserum (T-3003, Wien Laboratories). Standards from 0.5 to 125 pg were run in triplicate; samples were run in duplicate, averaged, and corrected for individual recovery. All yolk samples were run in one assay (intra-assay coefficient of variation, CV = 7%). Plasma samples, including those for adult males in Jenssen *et al.* (in press), were run in four assays (intra-assay CV = 14%; inter-assay CV = 11%). Nondetectable samples were assigned the least detectable value (0.5 pg per sample tube for all assays).

Experimental T Elevation and Behavior Trials

In July of 1999, we collected 26 juvenile *A. carolinensis* (14 males, 12 females), 36- to 42-mm SVL, from the field and brought them to the laboratory for use in behavior trials. Eight males and six females were randomly assigned to the T-implant group, and the remaining six males and six females were assigned to the blank-implant (control) group. Implants were made from Silastic tubing (Dow Corning; i.d. = 1.47 mm, o.d. = 1.96 mm) cut to a total length of 2–3 mm. T implants contained approximately 0.5 mm packed crystalline T (Sigma), and blank implants were empty. Both T and blank implants were closed at the ends with silicone sealant (Dow Corning). Within 4 days of being in the laboratory, all lizards were implanted subcutaneously, dorsolateral to the right hind leg, through a small incision in the skin that was closed with Vetbond tissue adhesive (3M). Lizards were cooled on ice for 5 min prior to surgery and were

TABLE 1
Behaviors, Definitions, and Point Values Used to Create a Behavior Index (BI) for Juvenile Male and Female *Anolis carolinensis*

Behavior	Definition	Point value
Head-up[a]	Posture reflecting alertness to the environment; >60 s (consecutively) with head raised higher than body	1
Perch shift	Any movement >1 body length (excluding tail) from one perch site to another; movements >15 s apart were scored as separate perch shifts	2
Tongue touch[b]	Potential chemosensory behavior involving brief touch of the tongue to the substrate	3
Color change[a,b]	Change in lizard body color between green, olive, or brown as a potential indicator of social stress or arousal	4
Headbob[c]	Series of vertical head movements in species-specific temporal cadences used for communication; noted display type (A, B, C), separation distance between displaying lizards, and whether dewlap extension also occurred	5
Eyespot[a,d]	Development of dark spot posterior to each eye indicating increased adrenergic activity	6
Engorged throat[a,b]	Display modifier in which the ventral throat area remains enlarged	7
Sagittal expansion[a,b]	Display modifier in which the lateral view of the lizard becomes enlarged	7
Approach/retreat	A perch shift directly toward or away from another lizard when the separation distance is <30 cm	8
Attack	Lunge toward another lizard, within 10 cm, with an attempt at or actual physical contact such as biting	9

[a] These behaviors were scored a maximum of once per pairwise interaction trial for each lizard.
[b] Greenberg (1977).
[c] Following descriptions in DeCourcy and Jenssen (1994); Lovern (2000a).
[d] Hadley and Goldman (1969).

returned to their home cages immediately following surgery.

To examine differences in behavior among sex and treatment groups (T and control), we videotaped juveniles 14–21 days postimplant. We ran 14 T trials (6 male–male, 4 female–female, 4 male–female contexts) and 12 control trials (4 male–male, 4 female–female, 4 male–female contexts) by moving lizards to observation cages set up identically to housing cages, except that the plywood fronts were replaced with screens to permit observations. Prior to the trials, lizards were isolated by opaque, removable partitions. At 16–24 h after lizards were moved to the observation cages, trials were conducted by videotaping pairs of lizards for 15 min individually and then 15 min together following the removal of the partition. All interacting pairs had SVL measurements within 2 mm of one another and were always from the same treatment group. After the 30-min trial, the lizards were again separated by replacing the partition. The following day this procedure was repeated with different pairings. Thus, each lizard was observed in two trials in which it was exposed to a different lizard each time. Because cages were made of nonreflective materials, we could ensure that lizard responses were not influenced by their own reflections. We videotaped trials from a darkened blind, using a Panasonic AG 460 video camera fitted with an Aztec video telephoto converter (2.0X), and we subsequently analyzed videotapes by recording variables to preprinted checksheets. On the day of its last trial, each lizard was sacrificed and 15–25 μl of plasma was collected for confirming T concentrations in T- and blank-implanted lizards, although this protocol could give an elevated measure, relative to baseline, of T in the blank-implanted lizards (e.g., due to recent social interaction; Wingfield, Hegner, Dufty, and Ball, 1990). We also confirmed that each lizard still had its implant. Plasma was handled and analyzed by RIA as described above. The samples were run in one assay (intra-assay CV = 6%).

To quantify behavior among trials from different treatment groups and social contexts, we used a behavior index (BI) modified from Ortiz and Jenssen (1982) that represented behavioral intensity. Each of the behaviors in the BI was assigned a point value that increased with increasingly socially motivated behaviors (Table 1). We calculated BIs by summing the points of the observed behaviors for each lizard individually (individual BIs) before partition removal and also for each lizard (when examining characteristics of individual behavior) or for each pair of lizards in a trial (trial BIs; when examining characteristics of trials, e.g., differences in overall behavioral intensity among social contexts) following partition removal.

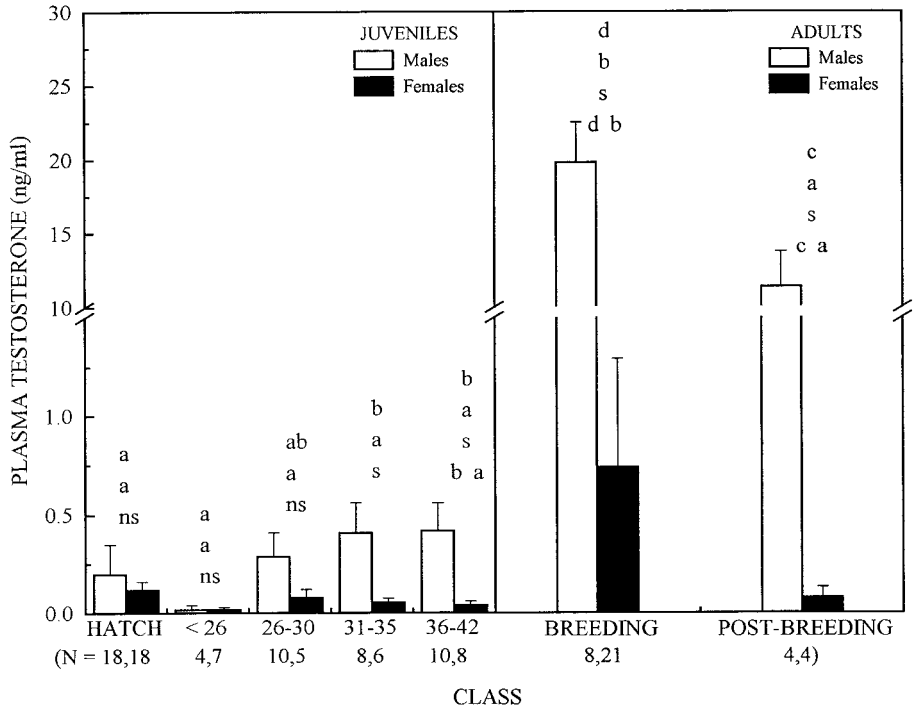

FIG. 1. Mean (+ 1 SE) plasma testosterone (T) concentrations for 50 juvenile male and 44 juvenile female *Anolis carolinensis* by size class (snout–vent length, mm) and for 12 adult male and 25 adult female *A. carolinensis* by season (sample sizes in parentheses). The adult male data are from Jenssen *et al.* (in press). The first juvenile class ("Hatch" denotes day of hatching) was sampled in the laboratory; all other classes were sampled in the field. Kruskal–Wallis test; $H_{13} = 57.8$, $P < 0.0005$. Results of nonparametric multiple comparisons are shown above the bars. Comparisons are valid only within rows; size and / or reproductive classes that do not share a letter designation are statistically different. Row 1, comparisons of males by class; 2, comparisons of females by class; 3, within-class comparisons of males and females (s, significantly different; ns, not significantly different); 4, comparisons of 36- to 42-mm juveniles and breeding and postbreeding adults of both sexes.

Statistical Analyses

Yolk T concentration was normally distributed (Kolmogorov–Smirnov test; $P > 0.15$) and analyzed by general linear model ANOVA, with hatchling sex and mother as main effects. However, endogenous plasma T concentration was not normally distributed ($P < 0.02$) and data transformation did not result in a normal distribution. Therefore, we used nonparametric Kruskal–Wallis tests to examine plasma T differences.

For behavior trials, we used Wilcoxon signed-ranks tests to compare paired BIs for individuals when alone versus in interactions (averaged from the two trials in which each lizard participated), and we used Kruskal–Wallis tests to examine main effects (treatment, social context, or sex) on BIs. Trial BIs were statistically independent of one another because each pair of interacting lizards was unique. When our objective was to compare specific behaviors among treatments, we averaged responses of individuals and examined across individuals using Fisher's exact tests, χ^2 tests, or Kruskal–Wallis tests. Descriptive statistics are reported as means ± 1 SE. We used Minitab (version 10Xtra) for statistical analyses, and hypothesis tests were two-tailed with $\alpha = 0.05$.

RESULTS

Endogenous Plasma T Concentrations

Plasma T concentrations significantly differed by class ($H_{13} = 57.8$, $P < 0.0005$; Fig. 1). Therefore, we used rank-based multiple comparisons (Hollander and Wolfe, 1973) to examine the relevant subset of all possible pairwise comparisons (see Fig. 1). Juvenile males generally showed increasing plasma T concentrations with size class, as lizards with SVLs of 31–42 mm (size classes 4 and 5) had significantly higher T than lizards in size classes 1 and 2 (hatchlings and lizards <26 mm from the field). Furthermore, for size classes 4 and 5, juvenile males had significantly higher plasma T concentrations than juvenile females. Breed-

ing adult males had higher plasma T concentrations than any other class, followed by postbreeding adult males. Adult females also had detectable plasma T, which, as in males, was higher in the breeding than in the postbreeding season.

Yolk T Concentrations

All eggs sampled contained detectable yolk T on the day of oviposition. However, the eggs that gave rise to males had significantly higher yolk T concentrations than those that gave rise to females ($F_{1,18} = 7.9$, $P = 0.02$). Yolk T content was 0.92 ± 0.09 and 0.51 ± 0.07 pg/mg in eggs giving rise to males and females, respectively. There was no relationship between mother and yolk T concentration ($F_{7,18} = 0.9$, $P = 0.52$).

Experimental T Elevation and Behavior Trials

Juveniles with T implants had significantly higher plasma T concentrations than those with blank implants ($H_3 = 19.6$, $P < 0.0005$). T-implanted males and females had 33.6 ± 2.0 and 34.4 ± 5.0 ng/ml T, respectively, whereas blank-implanted males and females had 0.71 ± 0.2 and 0.02 ± 0.01 ng/ml T, respectively. Multiple comparison procedures indicated that T-implanted males and females did not significantly differ, but that males and females with blank implants did, in plasma T concentrations. Furthermore, plasma T concentrations of blank-implanted males and females were similar to those of identically sized (36- to 42-mm SVL) juvenile males (0.42 ± 0.14 ng/ml) and females (0.04 ± 0.03 ng/ml) sampled in the field ($H_1 = 0.9$, $P = 0.36$, and $H_1 = 0.1$, $P = 0.83$, respectively, for the comparisons between laboratory and field males and females). The mean plasma T concentration for T-implanted juvenile lizards was 1.7× higher than the mean, but within the range, of plasma T concentrations for breeding adult males in the field (19.8 ± 2.7 ng/ml; minimum = 12.7, maximum = 36.7).

All lizards, regardless of sex or treatment, had higher behavioral intensities during interactions than when alone ($Z = 351$, $P < 0.0005$). Trial BIs were not affected by whether encounters were male–male, female–female, or male–female, for either blank-implanted ($H_2 = 1.0$, $P = 0.62$) or T-implanted lizards ($H_2 = 2.5$, $P = 0.29$), but trial BIs from the T-implanted group were substantially higher than those from the blank-implanted group (313 ± 45 vs 99 ± 36, respectively; $H_1 = 11.9$, $P = 0.001$).

We examined individual responses to experimentally elevated T by averaging individual BIs across the two trials in which juveniles participated. BIs were significantly higher in T-implanted than in blank-implanted juveniles, both for individuals while they were alone ($H_1 = 5.3$, $P = 0.02$, Fig. 2a) and during interactions ($H_1 = 10.8$, $P = 0.001$, Fig. 2b). Neither BIs for isolated lizards nor BIs for interacting lizards were affected by sex ($H_1 = 1.4$, $P = 0.23$; $H_1 = 1.1$, $P = 0.29$, respectively).

The higher BIs of T-implanted lizards, compared to blank-implanted lizards, were the result of increases in rates or probabilities of many of the measured behaviors in the T-implanted group. No behavior decreased in expression in the T-implanted lizards; rather, all behaviors remained at the level, or higher than that, of blank-implanted lizards. Within treatment group, there was no sex difference in display rate (Fig. 3). However, display rates of T-implanted juveniles were nearly ninefold higher than those of blank-implanted juveniles, and this difference was sig-

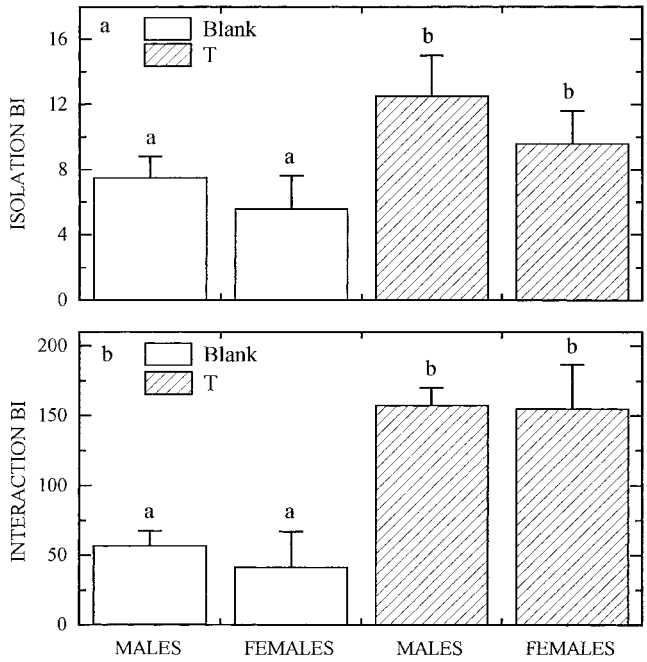

FIG. 2. Mean (+ 1 SE) individual (a) isolation and (b) interaction behavior indices (BIs) of juvenile *Anolis carolinensis* for six males and six females that received blank implants and for eight males and six females that received testosterone (T) implants. Isolation and interaction BIs represent behavior levels of individuals when alone and after being introduced to another lizard, respectively. The BIs from the two trials in which each lizard participated were averaged to create their individual BIs. Note the very different scales on the *y*-axes for (a) and (b). Different letters above bars indicate statistical significance after nonparametric multiple comparisons following Kruskal–Wallis tests and are applicable only within each section of the figure.

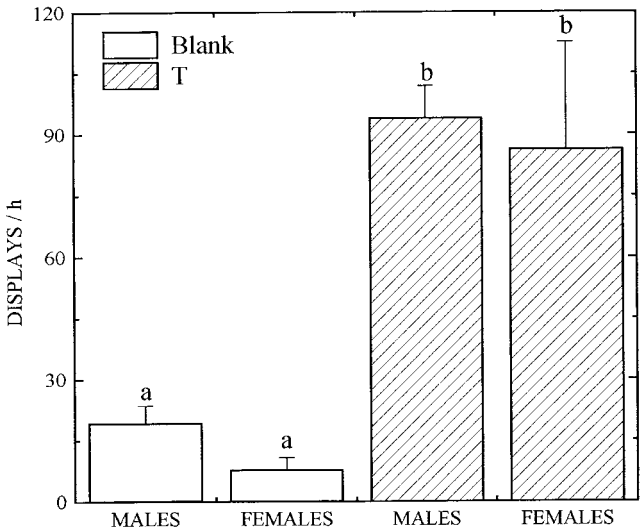

FIG. 3. Mean (+ 1 SE) headbobbing displays per hour during pairwise interactions of juvenile *Anolis carolinensis* for six males and six females that received blank implants and for eight males and six females that received testosterone (T) implants. Kruskal–Wallis test: $H_3 = 15.7$, $P = 0.001$; different letters above bars indicate statistical significance after nonparametric multiple comparisons.

nificant ($H_3 = 15.7$, $P = 0.001$; Fig. 3). Furthermore, a higher proportion of T-implanted than blank-implanted juveniles gave headbobbing displays with dewlap extension, used display modifiers, displayed in isolation, showed body color changes, developed an eyespot, and approached or retreated from the other lizard in an interaction (Table 2). All blank- and T-implanted lizards used the head-up posture, and perch shifted at least once, during interactions.

In addition to the display rate differences described above, the relative proportions of display types used differed between treatment groups ($\chi^2_2 = 7.0$, $P = 0.03$). Blank-implanted juveniles gave 17% type A displays, 13% type B displays, and 70% type C displays. In contrast, T-implanted juveniles gave 34% type A displays, 25% type B displays, and 41% type C displays. Both sexes responded to T implants by increasing the relative proportions of display types A and B in relation to type C ($\chi^2_2 = 14.0$, $P = 0.001$; $\chi^2_2 = 50.5$, $P < 0.0001$, for males and females, respectively). Nevertheless, T-implanted males and females differed in relative display proportions ($\chi^2_2 = 39.2$, $P < 0.0001$), but blank-implanted males and females did not ($\chi^2_2 = 2.0$, $P = 0.37$). T-implanted males gave 19% type A, 28% type B, and 53% type C displays, and T-implanted females gave 43, 28, and 29% type A, B, and C displays, respectively.

Although T-implanted juveniles, regardless of sex, exhibited more types and higher rates of many behaviors, some behaviors were conspicuously absent. No physical contact ever occurred between interacting lizards, although approaches and retreats (movements directly toward or away from the other lizard when interacting at a distance of <30 cm) occurred in 33% (4 of 12) and 36% (5 of 14) of control and T trials, respectively. We did not observe adult male-typical ritualized aggression (approach–circle–jaw spar–jaw lock) in any trial, nor did we observe courtship / reproductive behavior in the form of adult male-typical copulatory attempts or adult female-typical neck-bending.

TABLE 2

Proportion of Blank- and Testosterone (T)-Implanted Juvenile Male and Female *Anolis carolinensis* Expressing Specific Behaviors (Definitions in Table 1), and *P* values for Fisher's Exact Tests on Effect of Treatment

Behavior	Blank implant (6 males, 6 females)	T implant (8 males, 6 females)	*P*
Tongue touch			
Males	4/6	6/8	0.420
Females	3/6	5/6	0.243
Combined	7/12	11/14	0.185
Headbobbing display			
Males	6/6	8/8	1.000
Females	5/6	6/6	0.500
Combined	11/12	14/14	0.463
Displays with dewlap			
Males	5/6	8/8	0.429
Females	1/5	5/6	0.039
Combined	6/11	13/14	0.017
Displays in isolation			
Males	0/6	4/8	0.069
Females	0/6	1/6	0.500
Combined	0/12	5/14	0.030
Display modifier—			
Engorged throat			
Males	3/6	6/8	0.279
Females	1/6	6/6	0.008
Combined	4/12	12/14	0.009
Display modifier—			
Sagittal expansion			
Males	3/6	7/8	0.160
Females	2/6	5/6	0.114
Combined	5/12	12/14	0.023
Body color change			
Males	4/6	7/8	0.329
Females	2/6	6/6	0.030
Combined	6/12	13/14	0.020
Eyespot			
Males	4/6	6/8	0.419
Females	0/6	6/6	0.001
Combined	4/12	12/14	0.009

DISCUSSION

Endogenous Plasma T Concentrations

The present study is the first, to our knowledge, to document endogenous T concentrations during ontogeny in a natural population of lizards. We found that both juvenile male and female *A. carolinensis* had detectable plasma T and that T concentrations were always the same or greater in juvenile males than in juvenile females. Plasma T concentrations in juvenile males were in the range of those of breeding adult females (<1 ng / ml), but were considerably lower than those of breeding (20 ng / ml) or even postbreeding (11 ng / ml) adult males. Juvenile females had plasma T concentrations comparable to those of postbreeding females (<0.1 ng / ml) and less than those of breeding females.

The changes in plasma T concentrations in juveniles during ontogeny suggest the following interpretation. During the first 2 weeks after hatching, juveniles tend to have declining plasma T concentrations. If hatchling T primarily comes from maternal T deposited into the yolk and absorbed by the developing embryo (see below), this posthatching decline suggests net T degradation, rather than net T production, in hatchlings of both sexes. After 14 days posthatching, endogenous T production in males probably begins to exceed T degradation, since plasma T concentrations increase about 4-fold. For the remainder of the juvenile growth period, male plasma T is 3- to 10-fold greater than that of size-matched females, although it remains 20- to 45-fold less than that of adult males.

Alternatively, the comparatively high plasma T concentrations in hatchlings could be due to incubation conditions or stress in the laboratory (all other juveniles were sampled in the field). However, there are at least two reasons that a laboratory effect is unlikely. First, laboratory housing and other potential stressors generally cause a reduction of endogenous T, not an increase, in a wide variety of vertebrates including reptiles (e.g., Greenberg and Wingfield, 1987; Moore *et al.*, 1991; Moore, Lemaster, and Mason, 2000). Second, in the present study we found that blank-implanted juveniles housed in the laboratory for several weeks had the same plasma T concentrations as juveniles of the same size sampled in the field.

Yolk T Concentrations

That newly oviposited *A. carolinensis* eggs contain yolk T opens the door for an endocrine-mediated maternal effect on offspring phenotype, an area of research with exciting prospects (e.g., Birkhead, Schwabl, and Burke, 2000). Maternally derived T has been documented in the egg yolks of birds (e.g., Schwabl, 1993; Gil, Graves, Hazon, and Wells, 1999; Lipar, Ketterson, and Nolan, 1999), turtles (with both TSD and GSD; Janzen, Wilson, Tucker, and Ford, 1998), and alligators (with TSD; Conley, Elf, Corbin, Dubowsky, Fivizzani, and Lang, 1997). The amount of yolk T available to a developing embryo can affect posthatching growth and behavior (canaries; Schwabl, 1993, 1996), and T may be deposited in higher concentrations when females are mated to higher quality mates (zebra finches; Gil *et al.*, 1999).

To our knowledge the present study is the first to report a sex difference in yolk T concentrations; specifically, T was higher in eggs containing male embryos than in those containing female embryos. We assumed that yolk T at oviposition would be of maternal origin, because neither the gonads nor the adrenal glands (a possible extragonadal source of T) have undergone morphological differentiation at oviposition in lizards (e.g., *A. carolinensis;* Forbes, 1956; *Sceloporus undulatus;* Austin, 1988). However, it is not known whether the morphologically undifferentiated, presumptive gonads and adrenal cortices are steroidogenic.

That breeding females might differentially allocate T to developing males and females is surprising and difficult to explain. Anoline lizards have the unusual trait of laying single-egg clutches, alternately produced by the left and right ovary (Smith, Sinelnik, Fawcett, and Jones, 1973). If plasma T concentrations vary from one ovulatory cycle to the next within females, then differences in yolk T concentrations among eggs could result, although how mothers could detect embryonic sex and differentially deposit T based on this information remains unclear.

Differential allocation of T by mothers is not the only possible explanation for the observed sex difference in yolk T, however. In comparison to avian embryos, reptilian embryos are well developed at oviposition. *Anolis* embryos at oviposition, like those of most oviparous lizards, are at approximately stage 30 in the 40-stage embryonic development sequence developed by Dufaure and Hubert (1961) (Robin Andrews, personal communication), or approximately 20% through the time period between fertilization and hatching (the characters on which the developmental stages are based do not arise linearly by time). Thus, instead of differential T input by mothers, sex differences in yolk T at oviposition could arise by differen-

tial embryonic production of T or metabolism of maternally derived T. To determine the source of yolk T in *A. carolinensis* eggs, future studies should attempt to replicate the sex difference in yolk T at oviposition and measure yolk T content and potential embryo steroidogenesis at different stages of embryonic development.

Effects of T on Behavior

Our data for the effects of experimentally elevated T concentrations on juvenile behavior and the endogenous T data discussed above are together most consistent with the hypothesis that T activates, but does not organize, many adult male-typical behaviors in *A. carolinensis.* Juvenile males were exposed to higher natural levels of T than juvenile females, beginning possibly during incubation and then again after approximately 14 days posthatching. Such a pattern of differential T exposure between males and females is consistent with the possibility of an organizational effect on behavior followed by later activational effects when T varies seasonally in adults (Tokarz *et al.,* 1998; Jenssen *et al.,* in press). However, T implants caused similar or identical behavioral responses in juvenile males as well as females, thus suggesting that early T exposure had little effect on the behaviors produced by older juveniles. Juvenile males and females given T implants, but not those given blank implants, had markedly increased activity levels that approached those of breeding adult males tested under similar conditions (e.g., DeCourcy and Jenssen, 1994; Jenssen *et al.,* 2000). Behaviors affected by T in the present study included headbobbing display rate (both while alone and during interactions) and the relative proportion of display types (A, B, and C) used, dewlap and display modifier use, body color change, and eyespot development. These results were consistent across social contexts (consexual and heterosexual interactions). The observation that some T-implanted juveniles displayed while isolated from other lizards was especially dramatic. None of the 12 blank-implanted juveniles displayed while alone, and in over 100 h of observations on untreated juveniles in a separate study, no displays were ever observed from lizards while they were alone, although nearly all of them displayed during interactions (Lovern, 2000a).

The observation that juvenile males and females given T implants had higher activity levels even when they were not in interactions documents the extent of the effect of T on behavior. The stimulus of the presence of another lizard was not required to observe increased behavioral activity. Similar effects of T on general activity levels have been reported in adult males of several other species, including lizards (e.g., Marler and Moore, 1989; DeNardo and Sinervo, 1994; Klukowski, Jenkinson, and Nelson, 1998) and birds (e.g., Chandler, Ketterson, Nolan, and Ziegenfus, 1994). Functionally, increased activity results in larger and more actively patrolled home ranges and probably increased reproductive success through greater access to breeding females or greater ability to thwart intruding reproductive males (Marler and Moore, 1989; Chandler *et al.,* 1994; DeNardo and Sinervo, 1994). However, there can be a cost to high T, as experimental elevation of plasma T can lead to reduced growth rates and higher mortality (Marler and Moore, 1988, 1989; Hews, Knapp, and Moore, 1994; Abell, 1998; Klukowski *et al.,* 1998). Such a cost might be especially high in juveniles and may help to explain their comparatively low plasma T levels.

Although it apparently did not organize the behaviors we monitored, higher endogenous T concentrations in juvenile males than in juvenile females may affect morphological differentiation. For example, T exposure at appropriate concentrations and stages during ontogeny (in the plasma or yolk) might initiate morphological differentiation in postanal scale size, body length and mass, dewlap area, and underlying brain and peripheral structures associated with dewlap extension and courtship, all of which are sexually dimorphic in adults (Jenssen, Congdon, Fischer, Estes, Kling, and Edmands, 1995; Wade, 1998; O'Bryant and Wade, 1999; Jenssen *et al.,* 2000). Hatchlings develop postanal scale dimorphism just prior to hatching (Pearson and Licht, 1974), and although body length, mass, and dewlap area are not sexually dimorphic at hatching, they diverge during the course of posthatching ontogeny (Gordon, 1956; Crews and Greenberg, 1981; Michaud, 1990). Thus, the timing of the differentiation of these morphological traits, coupled with the endogenous T concentrations documented in the present study, suggest that embryonic exposure to T (whether maternal or embryonic in origin) may be responsible for differentiation of postanal scale size and that juvenile plasma T may be responsible for differentiation of body size and dewlap area. Further work on the exact timing of morphological differentiation between males and females and how it is affected by T will be necessary to examine these possibilities.

Although juveniles showed a dramatic behavioral response to T, several behaviors that are seen in adults were not seen in juveniles. First, unlike adult males,

there was no evidence of ritualized aggression. The behaviors observed in juveniles given T implants suggest increased patrolling, territory advertisement, and heightened agonistic responses to other lizards. However, less than half of the interactions yielded close (<30 cm) approaches, and none led to circling, jaw sparring, jaw locking, or indeed any physical contact. It is possible that longer interactions may have led to these behaviors, as we observed lizard pairs for only 15 min. However, this seems unlikely because responses tended to be intense but brief, typically declining prior to the end of the trial. By the 15-min point, none of the interactions that we observed appeared to be progressing in intensity, suggesting that the duration of the trial was not the main reason that ritualized aggression was not observed. Other potentially important factors could include residence time in the cage (e.g., Crews, 1980), length of exposure to T, and previous experience, each of which may have affected the probability of observing ritualized aggression. Second, copulation was never observed in juvenile interactions. Juvenile females never gave the characteristic neck-bend posture indicating receptivity. In contrast, gonadectomized adult females that are given T can exhibit either masculine courtship or feminine receptivity, the latter mediated by conversion of T to estradiol (Adkins and Schlesinger, 1979; Winkler and Wade, 1998). Although copulation was not observed and our sample size was small, interactions between T-implanted juvenile male and female *A. carolinensis* did suggest at least attempted courtship by males. In three of four male–female interactions, males appeared to initiate the interaction by courting the female. Typical of courtship interactions, these males displayed toward the females, extending their dewlaps while approaching steadily. Aggressive intent appeared to be absent because no display modifiers were employed at this point. However, the females in each case immediately responded with aggressive display behavior, using their dewlaps and employing display modifiers. In response, each of the males became aggressive as well, rapidly developing an eyespot, exhibiting a change in body color from green to dark brown, and employing aggressive display modifiers. Thus, the interaction clearly finished in an aggressive context, although it may have begun (from the male's perspective) as a courtship interaction. Because both ritualized aggression and copulation require coordinated social responses between interacting lizards, the relationship between sex steroids, individual behavior, and emergent behavioral interactions among individuals likely depends on additional ontogenetic factors, both physiological and experiential.

In conclusion, our data document that: (1) juvenile male and female *A. carolinensis* have detectable T in their plasma after hatching and in the yolks of the eggs from which they hatch; (2) juvenile males have significantly higher plasma T concentrations than juvenile females by the time they have SVLs of >30 mm (approximately 38 days posthatching); and (3) experimentally elevated plasma T concentrations produce increased expression and rates of behaviors in both juvenile males and females, and the level of these behaviors is similar to that seen in breeding adult males. These data are most consistent with the hypothesis that T activates, but does not organize, adult male-typical behavior. Thus, sexual dimorphisms in behavior in adults likely arise through underlying physiological differences between males and females that mediate the expression of behavior, rather than through fundamental sex differences in the ability to perform sexually dimorphic behaviors.

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