The role of 5α-reductase activity in sexual behaviors of the green anole lizard

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Received 26 October 1999; received in revised form 7 December 1999; accepted 29 December 1999

Abstract

Both testosterone (T) and its metabolite, 5α-dihydrotestosterone (DHT), can facilitate male sexual behavior in the lizard Anolis carolinensis. The present study addresses the role of DHT synthesis in regulating male sexual behavior by inhibiting 5α-reductase, the enzyme that converts T into DHT. In two separate experiments (one replacement and one maintenance paradigm), breeding adult males were castrated and implanted with capsules of T, DHT, or a control capsule (blank, BL). The animals were then injected with the 5α-reductase inhibitor, FCE, or with steroid suspending vehicle (SSV) as a control. Both experiments produced similar results. Overall, T was most effective in eliciting courtship and copulatory behaviors above control levels. In both experiments, treatment with FCE attenuated the T-induced effects on courtship behavior, whereas the inhibition of 5α-reductase activity resulted in modest and inconsistent effects on the latency to intromission and the proportion of copulating males. DHT treatment did not significantly increase courtship or copulatory behaviors above control levels. These results suggest that (a) 5α-reductase activity is necessary but that DHT alone is not sufficient for stimulating courtship in male A. carolinensis; and (b) courtship behavior is more sensitive than copulatory behavior to the activity of the androgen metabolizing enzyme. © 2000 Elsevier Science Inc. All rights reserved.

Keywords: Steroid hormone; Sexual behavior; Steroid metabolism; Lizard

1. Introduction

The metabolism of testosterone (T) plays an important role in activating male sexual behaviors. In the brain, the aromatase enzyme converts T into 17β-estradiol (E2), and the 5α-reductase enzyme converts T into 5α-dihydrotestosterone (DHT). However, the relative importance of the two metabolites of T varies with species and type of behavior. For example, in rats [7,12] and Japanese quail [57], the conversion of T to E2 (aromatization) is required for complete expression of masculine copulatory behaviors, whereas DHT is ineffective in activating the behaviors in these species [23,37,52]. In many other vertebrate species, T metabolism into androgenic steroids has been shown to facilitate male sexual behaviors, such as calling in the green tree frog [46] and African clawed frog [58], ultrasonic vocalizations in the Swiss–Webster mouse [44] and deer mouse [48], scent-marking behavior in the gray short-tailed opossum [15], chin marking in the New Zealand rabbit [20], and chemoinvestigatory behavior in the hamster [49,50].

As in other vertebrate species, male sexual behaviors are T-dependent in the green anole, Anolis carolinensis. In this species, males perform courtship displays, which consist of rapid head bobbing and extension of a large ventral throat fan [dewlap; 9,22,62]. If the courted female is receptive, the male will then clasp the skin on the back of the female’s neck with his jaws, mount, and intromit [9,22,62]. Hormone manipulation studies indicate that the expression of these courtship and copulatory behaviors does not require the aromatization of T (aromatase activity) because (a) peripheral administration of androgens [1,35,42] but not estrogen [11,35,62] can reinstate male sexual behaviors in castrated male anoles; and (b) inhibition of aromatase activity does not decrease these behaviors [62].

The role of neural 5α-reduction in male anoles is less clear. Experiments designed to indirectly test the importance of T metabolism into androgenic steroids have produced conflicting results. For example, although DHT has been shown to facilitate male sexual behavior [1], one study reported no effect of peripheral DHT administration alone [11]. Yet in the same study, DHT combined with E2 increased courtship behaviors in half of the individuals [11]. Implants of DHT directly into the anterior hypothalami-
preoptic area (AH-POA) also have restored courtship behaviors in castrated males [10]. Procedural differences probably account for some of the inconsistencies among these studies. DHT was effective in restoring behavior when lizards had been castrated for a relatively short period of time prior to onset of hormone treatment, [1 or 7 days; 1] and had been treated with hormone for a relatively long period of time prior to behavioral testing (14 days). In contrast, DHT failed to produce an effect when lizards were treated for only 2 days, starting 15 days after castration [11].

Because exogenous administration of DHT has produced inconsistent results, the present study was designed to directly address the behavioral importance of 5α-reductase activity (DHT synthesis) in A. carolinensis by inhibiting the activity of this enzyme with FCE 28260 [(22RS)-N-(1,1,-trifluoro-2-phenlprop-2yl)-3-oxo-4-aza-5α-androst-1-ene-17β-carboxamide; FCE, also called PNU 156765; Pharmacia and Upjohn]. This inhibitor was used because it dramatically reduces DHT production yet has no antiandrogenic effects in vivo [19]. In addition, the present study examined the effects of FCE and DHT in two different treatment paradigms in an attempt to reconcile the inconsistencies among previous experiments involving DHT.

2. Materials and methods

2.1. Housing and care

Adult A. carolinensis were purchased during the spring and summer breeding season from Buck’s or Fluker Farms in Louisiana. Because of space constraints, lizards were housed upon arrival in the lab either individually in 10-gallon aquaria or as groups in 29-gallon aquaria. The groups consisted of 1–2 males and at least 3 females. For the study, lizards were chosen randomly from these different conditions and housed individually during the experiment. Visual contact was prevented between experimental males by placing a black Plexiglas divider between adjacent cages. Stimulus females were housed together in a 29-gallon aquarium without the presence of a male. Lizards were kept in a 14L:10D photoperiod and had access to a heat lamp placed directly over each cage. They were sprayed daily with water and provided with cricket bites three times per week. Water in a bowl was provided ad libitum. Prior to all surgeries, animals were deeply anesthetized with Isofluorane. All procedures were in accordance with guidelines of the National Institutes of Health and the Michigan State University Animal Use and Care Committee.

2.2. Experimental design

2.2.1. Overall design

Two experiments were conducted. The first study investigated the ability of testosterone to restore sexual behavior when 5α-reductase activity was inhibited. On the basis of those and previous results (see Introduction and below), greater doses of hormone were administered in a maintenance paradigm in the second experiment.

The time frame for all procedures is outlined in Fig. 1. Before Experiment 1, males were tested to insure that they would display courtship behaviors in the lab (see following section on behavior testing). Males that displayed were gonadectomized and then immediately housed individually in 10-gallon aquaria. After 1 week, pretests for sexual behavior were conducted on 2 consecutive days to confirm that the behaviors were few and statistically equivalent across treatment groups. Following the second pretest, lizards were implanted subcutaneously with one Silastic capsule filled with testosterone propionate (TP), dihydrotestosterone propionate (DHTP), or sesame oil (blank, BL). Beginning 1 week after receiving the implants, males were injected subcutaneously for 7 days with the 5α-reductase inhibitor, FCE.
or with steroid suspending vehicle (SSV; for recipe, see [55]). The posttests were conducted on each of the last 3 days of injections.

For Experiment 2, males were castrated and immediately implanted with a subcutaneous capsule that contained TP or DHT, or with one that was empty (blank, BL). Starting the following day, lizards received daily subcutaneous injections of FCE or SSV for 28 days. Behavior tests were conducted once per week, beginning 7 days from the first injection. Following the second behavior test, the capsules were replaced in all males to insure that they contained a sufficient amount of hormone throughout the study. Upon removal, all hormone-containing capsules were at least one-third full. In both experiments, lizards were sacrificed following the last behavior test to obtain tissue for assaying effectiveness of the experimental treatments.

2.2.2. Treatments

A total of five treatment groups (eight males per group) were created in Experiment 1: (a) BL + SSV, (b) BL + FCE, (c) DHTP + SSV, (d) TP + SSV, and (e) TP + FCE. For Experiment 2, males were assigned to the following five treatment groups: (a) BL + SSV (n = 8), (b) DHTP + SSV (n = 8), (c) DHTP + FCE (n = 8), (d) TP + SSV (n = 7), and (e) TP + FCE (n = 9). The BL + FCE group was not included in the second experiment because in the first experiment, it was comparable to the BL + SSV group for all measurements. A total of 10 males died before termination of Experiment 2. To control for possible behavioral effects of declining health, these males were eliminated from all analyses. The final sample sizes for each treatment group in Experiment 2 were seven males for the BL + SSV, TP + SSV, and DHTP + SSV groups; five males for the TP + FCE group; and four males for the DHT + FCE group. All treatments were randomly assigned in both experiments.

The dimensions of the Silastic capsules used in the first study were 0.51 mm (inner diameter) × 0.94 mm (outer diameter) × 8 mm (length); in the second study, they were 0.76 mm (inner diameter) × 1.65 mm (outer diameter) × 7 mm (length). To facilitate packing into the small capsules used in Experiment 1, androgens were mixed with sesame oil at a ratio of 1 mg hormone per 750 μl oil for TP or 1.56 mg hormone: 750 μl for DHTP. In Experiment 2, capsules were packed with hormone only. All capsules were sealed at both ends with silicone medical adhesive (Dow Corning, Midland, MI). In a preliminary experiment, it was determined that a series of four daily injections of 30 μg FCE reduced 5α-reductase activity in the brain by 95% (measured 24 h after last treatment). Therefore, lizards in both experiments received daily injections of 30 μg FCE suspended in 20 μl of SSV or SSV alone as a control. All injections were administered between 0830 and 1130 h.

Stimulus females for the behavior tests were gonadectomized and injected with 4 μg of estradiol benzoate (EB) suspended in 20 μl of SSV, which activates receptivity in ovariectomized female A. carolinensis [35,38,51,62]. Injections were administered starting at least 24 h before the first day of behavior testing.

2.3. Behavior testing

Male courtship and copulatory behaviors have been described in detail elsewhere [9,22,62]. For the present experiments, a courtship bout was defined as a continuous succession of head bobs directed toward the stimulus female. In most cases, these bouts included extension of the dewlap. The numbers of courtship bouts and dewlap extensions were recorded during each behavior test. To quantify copulatory behavior, the latency to intromission and the number of males that intromitted per group were recorded in both experiments. These aspects of copulatory behavior were analyzed rather than frequency of copulation because the behavior typically occurs a maximum of once per test in this species under these conditions. In Experiment 2, latency to neck grip (the beginning of the copulation sequence) also was recorded so that rate of courtship displays could be calculated (males stop courting once copulation is initiated). If a male did not neck grip or intromit by the end of the 15-min test, the latency period for these behaviors was defined as 15 min.

In a few behavior tests, TP-treated males rapidly forced copulation with the female without courtship, which would not occur under natural conditions in the field. The omission of courtship was not due to an inhibitory effect of high T levels because it only occurred in one or two posttests of two TP-treated males. In the other posttests of the same males, frequency of courtship display was similar to the other males in the group. Hence, to obtain a more accurate representation of the effects of androgen treatment on courtship behavior in Experiment 1, the individual tests during which a male copulated within 5 min without courting were not included in the mean of posttest scores of courtship behaviors. In Experiment 2, only one test was conducted at each time point, so eliminating individual tests was not feasible. To be consistent with Experiment 1, the data were analyzed both with and without the one male (TP + FCE group) who copulated without courting in Experiment 2. In all but one case, statistical analyses were comparable with or without him, and the reported mean values include that data point. The statistics are reported both ways when exclusion of this male affected significance.

2.3.1. Prescreening behavior tests

Prior to Experiment 1, males housed in large cages with females were observed for 30 min on 2 consecutive days by an observer sitting at least 2 m away. Individually housed males were tested once by placing a stimulus female in the male’s home cage for 5 min. Only males that performed courtship bouts were used in the experiment. Most males achieved intromission by the end of the test.

2.3.2. Experimental behavior tests

In both studies, each male was presented with a stimulus female in his individual home cage. The behaviors were re-
corded for 15 min by an observer sitting behind a cardboard blind that was 1 m from the testing cages. The observer did not know the treatment condition of each lizard. For the consecutive behavior tests in Experiment 1, no male was presented with the same stimulus female. To facilitate viewing and minimize distractions, rocks and water dishes were removed from the cages prior to testing. The behavior of each animal was tested 1–4 h after the daily injection of FCE or SSV.

2.4. Tissue collection and processing

Following the last behavior test in both experiments, the lizards were rapidly decapitated. In Experiment 1, the brains were removed, flash frozen on dry ice, and stored at −80°C. In both experiments, kidneys were fixed in Bouin’s solution for later histological processing. After tissue collection, silastic capsules were examined to verify that hormone or oil still remained, as appropriate. Completeness of gonadectomy was confirmed at this time by inspection of the body cavity under a dissecting microscope. Because of incomplete gonadectomy, one lizard from the TP + FCE group in Experiment 1 was excluded from all analyses. As a measure of health for Experiment 2, body weight of the complete animal was measured immediately following decapitation.

2.4.1. 5α-reductase and aromatase activity

The assay for measuring activity of T-metabolizing enzymes was previously validated for anole brains [53]. Briefly, tissue from Experiment 1 was homogenized in sucrose phosphate buffer and incubated in duplicate with [3H]-T (New England Nuclear, 96.5 Ci/mmol) and with cofactors at 27°C for 50 min. The resulting steroid products were extracted with ether, and the androgens and estrogens were separated from each other by phenolic partition. Individual androgens and estrogens were further separated by thin-layer chromatography (TLC). The steroids of interest (i.e., T, E2, and DHT) were scraped from the TLC plates and eluted in aqueous methanol. Radioactivity associated with the steroid products was measured with a Beckman liquid scintillation counter (LS6500). Activity of 5α-reductase was quantified as the rate of conversion of T to DHT, and aromatase activity was quantified as rate of conversion of T to E2. Because of the limitation in the number of tubes that can be run in a single assay, five randomly chosen brains from the BL + SSV, TP + FCE, and TP + SSV groups (Experiment 1) were compared in one assay. Enzyme activities were corrected for protein content, which was measured with the Bradford method.

2.4.2. Kidney histology

Kidneys from both experiments were fixed in Bouin’s fluid for 7 days, dehydrated, embedded in paraffin, and sectioned at 10 μm. Sections were stained with hematoxylin–eosin. Androgen treatment stimulates the renal sex segment [11,42,62], a portion of the kidney that serves a function similar to the mammalian prostate. Therefore, as a bioassay of androgen exposure, the NIH image analysis program was used to measure epithelial cell height in the renal sex segment. A total of 16 measurements were made and averaged for each male (four cells in four randomly chosen tubules). To be sure of adequate hormone exposure, lizards were omitted from the study if their kidneys showed a low response to androgens (that is, if their mean epithelial height was more than two standard deviations below the mean of their respective treatment group). Two males in Experiment 1 (one each from the TP + SSV and TP + FCE groups) were eliminated from all behavioral and tissue analyses for this reason.

2.5. Statistical analysis

In Experiment 1, the number of dewlap extensions and courtship bouts were analyzed using an average of posttest scores. Main effects of treatment on courtship displays were determined with one-way ANOVAs, and pairwise comparisons were made using Fisher’s PLSD. Because duration of the courtship phase (i.e., latency to neck grip) was recorded in Experiment 2, the rates of dewlap extension and courtship bouts (number of behaviors divided by the latency to neck grip or by 15 min if no neck grip occurred) were analyzed for this experiment. Main effects of treatment and interactions of treatment with time were determined with repeated measures ANOVA. Differences among treatment groups at each time point were analyzed with one-way ANOVA, followed by Fisher’s PLSD.

Latency to intromission in both experiments was analyzed with the Kruskal–Wallis test because the data were not normally distributed. If a significant effect of treatment existed, pair-wise comparisons were made with the Mann–Whitney U-test. Group differences in the proportion of animals that copulated were analyzed with Fisher’s Exact 2 × 2 test. For Experiment 1, the proportion of copulators represents the number of animals that copulated at least once during the three posttests. For Experiment 2, the proportion of copulators was analyzed for each individual time point. Differences among treatment groups in T-metabolizing enzyme activity (Experiment 1) and renal sex segment stimulation (both experiments) were analyzed with one-way ANOVA and Fisher’s PLSD.

3. Results

3.1. Effectiveness of treatment

FCE acted as a potent and specific inhibitor of the 5α-reductase enzyme. In Experiment 1, the compound inhibited 5α-reductase activity in the brains of T-treated animals by 97%. (ANOVA: F(2, 12) = 39.52, p < 0.001; TP + SSV versus TP + FCE, Fisher’s PLSD p < 0.001; Table 1). The activity in the TP + SSV treated animals was similar to that previously reported for gonadally intact green anole lizards [53]. The inhibition was specific to 5α-reductase because
aromatase activity was not affected by FCE treatment. However, as typical for the aromatase enzyme [27], its activity was facilitated by treatment with androgen (ANOVA $F(2, 12) = 5.386$, $p = 0.051$; TP$^1$ SSV versus TP$^1$ FCE, Fisher’s PLSD: $p = 0.273$; TP$^1$ SSV versus BL$^1$ SSV, Fisher’s PLSD: $p = 0.017$). In both the TP$^1$ SSV and TP$^1$ FCE groups, aromatase activity was similar to those previously reported for intact green anoles [53].

The renal sex segment data indicate that androgen treatments were efficient and that FCE did not act either as an androgen agonist or antagonist. In both experiments, the renal sex segment was stimulated in the androgen treated animals relative to the controls (Experiment 1: ANOVA: $F(4, 31) = 19.02$, $p < 0.001$; Fisher’s PLSD: $p < 0.01$ for each androgen-treated group versus each BL-treated group, Fig. 2A; Experiment 2: ANOVA: $F(4, 26) = 19.37$, $p < 0.001$; Fisher’s PLSD: $p < 0.001$ for each androgen-treated group versus BL-treated group, Fig. 2B). Further, stimulation was equal in animals treated with the same androgen, regardless of FCE treatment (Experiment 1: TP$^1$ SSV versus TP$^1$ FCE Fisher’s PLSD: $p = 0.907$; Experiment 2: TP$^1$ SSV versus TP$^1$ FCE Fisher’s PLSD: $p = 0.667$; DHTP + SSV versus DHTP + FCE Fisher’s PLSD: $p = 0.165$). In both experiments, stimulation of the renal sex segment was generally comparable in DHTP and TP-treated animals, indicating that the levels of T and DHT produced by the pellets were sufficient to elicit similar physiological responses (Experiment 1: DHTP + SSV versus TP + SSV Fisher’s PLSD: $p = 0.391$; Experiment 2: DHTP + SSV versus TP + SSV Fisher’s PLSD: $p = 0.284$). In one case, DHTP appeared to be slightly more effective than TP (Experiment 2: DHTP + FCE versus TP + FCE Fisher’s PLSD: $p = 0.020$; Fig. 2B).

Although some animals died in Experiment 2, the proportion of males that died was not significantly different among individual treatment groups (chi square, $p = 0.167$). FCE did not appear to affect overall health of the remaining animals because their body weights were almost identical across treatment groups (ANOVA $F(4, 25) = 1.21$, $p = 0.332$; data not shown). Also, the general condition of each lizard was noted upon termination of the experiment, and no obvious differences were observed across treatment groups.

### Table 1

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>5α-reductase activity (mean ± SEM)*</th>
<th>Aromatase activity (mean ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BL + SSV</td>
<td>9.47 ± 2.27</td>
<td>0.28 ± 0.06</td>
</tr>
<tr>
<td>TP + SSV</td>
<td>17.95 ± 0.71</td>
<td>0.54 ± 0.07**</td>
</tr>
<tr>
<td>TP + FCE</td>
<td>0.57 ± 0.27</td>
<td>0.43 ± 0.07</td>
</tr>
</tbody>
</table>

*All means are significantly different from each other ($p < 0.05$).

**Units are fmoles per minute per milligram of protein.

**Mean is significantly different from BL + SSV group ($p < 0.05$).

BL indicates blank; SSV, steroid suspending vehicle; TP, testosterone propionate; FCE, FCE 28260.

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**3.2. Dewlap display**

**3.2.1. Experiment 1**

Overall, there was a statistically significant effect of treatment on dewlap displays (ANOVA: $F(4, 32) = 2.86$, $p = 0.039$; Fig. 3A). On average, dewlap extensions occurred more frequently in the TP + SSV group than in the control group, BL + SSV (Fisher’s PLSD: $p = 0.007$).
mean behavior display of the DHTP + SSV group was between that of the BL + SSV and TP + SSV groups and was not statistically different from either group (DHTP + SSV versus BL + SSV group, Fisher’s PLSD: $p = 0.082$; DHTP + SSV versus TP + SSV, Fisher’s PLSD: $p = 0.289$). Dewlap extension in the TP + FCE group was less than the TP + SSV group (Fisher’s PLSD: $p = 0.046$) and was not significantly different from the control group, BL + FCE (Fisher’s PLSD: $p = 0.600$).

3.2.2. Experiment 2

Results were consistent with the first experiment. When all tests were considered, there was a main effect of treatment on rate of dewlap display (repeated measures ANOVA: $F(4, 25) = 4.22, p = 0.010$), but not a significant effect of time ($F(3, 75) = 0.838, p = 0.477$) or an interaction of treatment with time ($F(12, 75) = 0.942, p = 0.510$; Fig. 4A). Overall, treatment with TP + SSV facilitated the rate of dewlap extension over control levels (collapsed across time; Fisher’s PLSD: $p = 0.001$). Further, TP + SSV appeared to be more potent than DHTP + SSV because the behavior overall was significantly more frequent in the TP + SSV group (Fisher’s PLSD: $p = 0.017$) and because the behavior in the DHTP + SSV group was not significantly more frequent than in the control group (Fisher’s PLSD: $p = 0.242$). Treatment with FCE appeared to inhibit T-facilitation of dewlap display, as the rate was lower in the TP + FCE compared to the TP + SSV group (Fisher’s PLSD: $p = 0.053$). Also, the behavior in the TP + FCE group was not significantly different from the control group (Fisher’s PLSD: $p = 0.171$).

When individual time points were considered, a significant main effect of treatment occurred at weeks 2 and 4, and a trend existed at week 3, which followed the replacement of the hormone capsule (ANOVA, week 1: $F(4, 25) = 0.815, p = 0.528$; week 2: $F = 2.98, p = 0.039$; week 3: $F(4, 25) = 2.51, p = 0.067$; week 4: $F(4, 25) = 3.31; p = 0.026$; Fig. 4A). At weeks 2–4, the rate of dewlap display in the TP + SSV group was significantly greater than in the BL + SSV group (Fisher’s PLSD week 2: $p = 0.004$; week 3: $p = 0.009$; week 4: $p = 0.002$). Treatment with FCE significantly inhibited the effects of TP at week 4 (Fisher’s PLSD: $p = 0.027$ with the male in the TP + FCE group who copulated without courting; $p = 0.064$ without him). Behavior was also generally lower in the TP + FCE than in the TP + SSV group during weeks 1–3, but the difference was not significantly different (Fisher’s PLSD: $p > 0.150$). Treatment with TP + FCE, DHTP + FCE, and DHTP + SSV were ineffective in facilitating dewlap display because the behavioral rates in these three groups did not differ significantly from those of the BL + SSV group at any time point (Fisher’s PLSD, week 2: all $p > 0.150$; week 3: all $p > 0.07$; week 4; all $p > .200$).

3.3. Courtship Bouts

3.3.1. Experiment 1

Overall, an effect of treatment existed (ANOVA: $F(4, 32) = 2.67, p = 0.050$; Fig. 3B). Courtship bouts were significantly greater in the TP + SSV group compared with the BL + SSV group (Fisher’s PLSD: $p = 0.012$). Treatment with the 5α-reductase inhibitor, FCE, inhibited the effects of TP on courtship behavior because courtship bouts in the TP + FCE group tended to be fewer than in the TP + SSV group (Fisher’s PLSD: $p = 0.052$) and were not significantly different from the control group, BL + FCE (Fisher’s
Fig. 4. Rate of dewlap extensions (A) and courtship bouts (B) for each group in Experiment 2. At week 1, all groups were statistically equivalent. By week 4, the frequencies of both behaviors were significantly higher in the TP + SSV group than in both BL + SSV and TP + FCE groups. The behaviors in the DHTP + SSV and DHTP + FCE groups were never significantly different from the behavior in the control group, BL + SSV. *Significantly different from the BL + SSV group (p < 0.05). **Significantly different from the BL + SSV and TP + FCE groups (p < 0.05).
PLSD: \( p = 0.582 \). Mean courtship display of the DHTP + SSV group was between the BL + SSV and TP + SSV groups and was not statistically different from either group (DHTP + SSV versus BL + SSV, Fisher’s PLSD: \( p = 0.092 \); DHTP + SSV versus TP + SSV, Fisher’s PLSD: \( p = 0.381 \)).

### 3.3.2. Experiment 2

The repeated measures ANOVA revealed a significant effect of treatment on rate of courtship display (\( F(4, 25) = 4.14, p = 0.011; \) Fig. 4B), but not an effect of time (\( F(3, 75) = 1.43, p = 0.240 \)) or an interaction of treatment with time (\( F(12, 75) = 1.43, p = 0.172 \)). Overall, courtship rate was significantly greater in the TP + SSV group, compared to the BL + SSV group (collapsed across time; Fisher’s PLSD: \( p = 0.001 \)). Further, TP + SSV appeared to be more potent than DHTP + SSV, since the behavior in the DHTP + SSV group was significantly less than the TP + SSV group (Fisher’s PLSD: \( p = 0.013 \)), and similar to the control group (DHTP + SSV versus BL + SSV Fisher’s PLSD: \( p = 0.332 \)). Treatment with FCE appeared to prevent the T-facilitation of courtship display, since the display rate was lower in TP + FCE−, compared to TP + SSV-treated animals (Fisher’s PLSD: \( p = 0.071 \)).

Similar to dewlap display, a main effect of treatment on courtship rate occurred on weeks 2 and 4, and a trend existed at week 3 (ANOVA week 1: \( F(4, 25) = 0.708, p = 0.594 \); week 2: \( F(4, 25) = 3.24, p = 0.029 \); week 3: \( F(4, 25) = 2.47, p = 0.071 \); week 4: \( F(4, 25) = 3.31, p = 0.026 \); Fig. 4B). During this time period, courtship rate was consistently greater in the TP + SSV group than in the BL + SSV group (Fisher’s PLSD, week 2: \( p = 0.003 \); week 3: \( p = 0.009 \); week 4: \( p < 0.001 \)). At week 2 only, the behavior also was significantly greater in the TP + FCE group compared to the BL + SSV group (Fisher’s PLSD, week 2: \( p = 0.025 \); week 3: \( p = 0.108 \); week 4: \( p = 0.209 \)). At week 4, courtship rate in the TP + FCE group was significantly less than the TP + SSV group (Fisher’s PLSD: \( p = 0.008 \); Fig. 4B). Treatment with DHTP + FCE or DHTP + SSV was ineffective in maintaining behavioral rates above control levels at all time points (Fisher’s PLSD: all \( p > 0.170 \) for each group versus BL + SSV).

### 3.4. Copulation

#### 3.4.1. Latency

In Experiment 1, a main effect of treatment on latency to intromission occurred (Kruskal–Wallis \( H = 11.51, p = 0.021; \) Table 2), such that it was lower in the three androgen treatment groups (TP + SSV, TP + FCE, DHTP + SSV) compared with those in their respective control groups, BL + SSV or BL + FCE (Mann–Whitney \( U \): all \( p < 0.050 \)). The latencies of the three androgen treatment groups also did not differ significantly from each other (Mann–Whitney \( U \): all \( p > 0.700 \)). Similar to Experiment 2, a main effect of treatment was detected at week 2 (Kruskal–Wallis \( H = 14.57, p = 0.006; \) Table 2) and again at week 4 (Kruskal–Wallis \( H = 13.68, p = 0.008 \), but not at weeks 1 (Kruskal–Wallis \( H = 7.29, p = 0.122 \)) or 3 (Kruskal–Wallis \( H = 6.95, p = 0.139 \)). At weeks 2 and 4, latency in the TP + SSV group was significantly less than that in the control group (Mann–Whitney \( U \): week 2: \( p = 0.025 \); week 4: \( p = 0.003 \)). Treatment with FCE had more subtle effects on the latency of TP-induced copulation. At week 2, the latency in this group was the same as that of the control group (Mann–Whitney \( U \): \( p > 0.999 \); Table 2), and tended to be greater than in the TP + SSV group (Mann–Whitney \( U \): \( p = 0.053 \)). However, by week 4, the latency in the TP + FCE group was significantly less than the control group, (Mann–Whitney \( U \): \( p = 0.025 \)). The time to intromission in the TP + FCE group was not significantly different than in the TP + SSV group at week 4, although FCE almost doubled the latency, on average (Mann–Whitney \( U \): \( p = 0.165 \); Table 2). At neither time point (week 2 or 4) did the DHTP + SSV or

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### Table 2

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Treatment group</th>
<th>Posttest (minutes)</th>
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<tbody>
<tr>
<td>1</td>
<td>BL + SSV</td>
<td>15.00</td>
</tr>
<tr>
<td></td>
<td>BL + FCE</td>
<td>15.00</td>
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<tr>
<td></td>
<td>DHTP + SSV</td>
<td>11.08 ± 1.90*</td>
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<tr>
<td></td>
<td>TP + SSV</td>
<td>10.48 ± 2.03*</td>
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<tr>
<td></td>
<td>TP + FCE</td>
<td>10.00 ± 2.05**</td>
</tr>
<tr>
<td>2</td>
<td>BL + SSV</td>
<td>Week 1: 15.00</td>
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<td></td>
<td></td>
<td>Week 2: 15.00</td>
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<tr>
<td></td>
<td></td>
<td>Week 3: 11.29 ± 2.40</td>
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<tr>
<td></td>
<td></td>
<td>Week 4: 13.31 ± 1.69</td>
</tr>
<tr>
<td></td>
<td>DHTP + SSV</td>
<td>Week 1: 15.00</td>
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<tr>
<td></td>
<td></td>
<td>Week 2: 15.00</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Week 3: 11.78 ± 3.22</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Week 4: 13.31 ± 1.69</td>
</tr>
<tr>
<td></td>
<td>DHTP + FCE</td>
<td>Week 1: 15.00</td>
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<tr>
<td></td>
<td></td>
<td>Week 2: 15.00</td>
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<tr>
<td></td>
<td></td>
<td>Week 3: 9.84 ± 2.40</td>
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<tr>
<td></td>
<td></td>
<td>Week 4: 5.52 ± 2.13</td>
</tr>
<tr>
<td></td>
<td>TP + SSV</td>
<td>Week 1: 10.68 ± 2.11</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Week 2: 9.05 ± 2.82*</td>
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<tr>
<td></td>
<td></td>
<td>Week 3: 8.48 ± 2.40</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Week 4: 5.52 ± 2.13</td>
</tr>
<tr>
<td></td>
<td>TP + FCE</td>
<td>Week 1: 15.00</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Week 2: 15.00</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Week 3: 9.94 ± 3.12</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Week 4: 9.19 ± 2.52</td>
</tr>
</tbody>
</table>

*Latency was defined as 15 min if copulation did not occur by the end of the test. Posttest latency in Experiment 1 is based on the average of the three post tests for each individual. For Experiment 2, the mean latency for each week is given.

*Significantly different from BL + SSV (\( p < 0.05 \)). Comparisons are within the same week for Experiment 2.

**Significantly different from BL + FCE (\( p < 0.05 \)).
DHTP + FCE groups differ significantly from the control group (Mann–Whitney U: all $p > 0.170$).

### 3.4.2. Proportion of males that copulated

In Experiment 1, only androgen-treated males copulated in the posttests (Table 3). The proportion of males that copulated during the posttests was similar in the three androgen-treated groups and identical in the two TP-treated groups. In these two groups, the proportion of males that copulated was significantly greater than the control groups, BL + SSV and BL + FCE (Fisher’s Exact: all $p = 0.03$). The proportion of males that copulated in the DHTP + SSV group was between that of the BL + SSV group and the TP + SSV group, and it was not significantly different from either group (Fisher’s Exact: DHTP + SSV versus BL + SSV, $p = 0.077$; DHTP + SSV versus TP + SSV, $p > 0.999$).

In Experiment 2, the proportion of males that copulated in the TP + SSV group was significantly greater than in the BL + SSV group, starting at week 2 (Fisher’s Exact, week 1: $p = 0.205$; week 2: $p = 0.035$; weeks 3 and 4: $p = 0.005$; Table 3). Treatment with FCE appeared to delay the effect of T on copulation because males in the TP + FCE group did not copulate until the last 2 weeks. Only at week 4 was the proportion of copulating males significantly greater in the TP + FCE group compared with that in the BL + SSV group (Fisher’s Exact, week 1: $p > 0.999$; week 2: $p > 0.999$; week 3: $p = 0.152$; week 4: $p = 0.05$). However, at no time point was the proportion of copulating males significantly different between the TP + FCE and TP + SSV groups (Fisher’s Exact, week 1: $p = 0.559$; week 2: $p = 0.081$; week 3: $p > 0.999$; week 4: $p = 0.522$). The proportion of copulators in the DHTP + SSV and DHTP + FCE groups were never significantly different from the number in the BL + SSV group (Fisher’s Exact, week 1: $p > 0.999$; week 2: $p > 0.999$; week 3: $p = 0.462$; week 4: $p = 0.999$).

### 4. Discussion

The results of both experiments suggest that in *A. carolinensis,* 5α-reductase activity is necessary for mediating the effects of T on male courtship behavior. However, 5α-reduced metabolites do not appear to act as prime facilitators of sexual behaviors. That is, DHT by itself (DHTP + SSV) did not significantly affect courtship behaviors in either the replacement or maintenance paradigm, but inhibiting 5α-reductase activity attenuated the effects of TP on courtship. The effects of FCE on copulation were more subtle and were limited to Experiment 2. Thus, 5α-reductase activity seems to be critical for facilitating both the activation and maintenance of courtship behaviors but plays a more minor role in the maintenance of copulation.

Similar to the present study, previous investigations of *A. carolinensis* indicate that T is the prime activator of male sexual behavior. In several studies, peripheral administration of T activated or maintained the behaviors [1,11,35,43,62], whereas the effects of DHT have been inconsistent. In one study, peripheral administration of DHT was as effective as T in facilitating sexual behaviors in castrated male *A. carolinensis* [1]. However, in a different study [11], peripheral treatment with either DHT or E2 failed to have a significant effect on behavior in castrated males, although treatment with the two hormones combined increased courtship behaviors in half of the individuals [11]. The present study is consistent with the results of Crews et al. [11] in that DHT alone did not significantly facilitate behavior above control levels. Furthermore, changing the treatment paradigm in the present study and increasing the size of the implant did not increase the efficacy of DHT, suggesting that it is less potent than T in facilitating masculine behaviors in green anoles.

The supporting role of 5α–reductase activity is not unique to courtship display in *A. carolinensis*; it has been observed in many vertebrate species. In mammals, full expression of the majority of sexual behaviors requires aromatization of T to E2, but 5α-reduction of T appears to facilitate some masculine behaviors. For example, in the male hamster, high doses of peripheral DHT can activate chemoinvestigatory behavior [49,50], and inhibition of 5α-reductase activity attenuates T-activation of the behavior [50]. The erectile response in rats is also dependent upon 5α-reductase activity. Treatment with DHT or T facilitates the response in castrated male rats [39], and treatment with a potent 5α-reductase inhibitor decreases the stimulatory effect of T [4]. Birds are similar to mammals in that the majority of male sexual behaviors require aromatization of T to E2, and DHT facilitates only some aspects of particular behaviors [2]. In contrast to these species and green anoles, 5α-reduced androgens appear to play a more central role in some ectother-
mic vertebrates. That is, DHT alone is sufficient to restore several masculine behaviors, including calling in the green tree frog [46] and African clawed frog [58], amplexic clamping in the African clawed frog [25] and newt [40], and copulatory postures in whiptail lizards [28,36,56].

The effects of 5α-reductase inhibition on sexual behaviors in A. carolinensis correlates with the distribution of the enzyme in the brain. The highest levels of 5α-reductase occur in the brainstem of A. carolinensis [53], which contains motor neurons that directly innervate the dewlap muscles [54]. Hence, in the present study, FCE most likely decreased courtship display by inhibiting 5α-reductase activity in the brainstem. However, steroid hormones also could act on other brain areas to stimulate sexual behavior, such as the anterior hypothalamic–preoptic area (AH-POA). This area appears to be important for both courtship and copulatory behaviors in male anoles, as lesions in this general area inhibit sexual behavior in lizards, including A. carolinensis [16,26,59]. The AH-POA appears to be sensitive to both androgens and estrogens. Although systemic administration of DHT or E2 is ineffective [11], implants of DHT or E2 directly into this area activate male courtship behavior in castrated A. carolinensis [10]. Also, the AH-POA of anoles contains binding sites for both androgens and E2 [34,41], and it has relatively high levels of aromatase activity [53]. This apparent E2 sensitivity of the AH-POA and the requirement of DHT in the brainstem might explain why systemic T consistently activates male sexual behaviors in numerous studies of A. carolinensis [1,11,35,42,62]; it serves as a source of both androgen and E2. This idea is not necessarily inconsistent with the ability of E2 or DHT implants in the AH-POA to facilitate courtship behavior. A high dose of either hormone directly administered to the AH-POA might compensate for a concurrent lack of DHT to the brainstem.

In addition to acting centrally, circulating DHT and T might act peripherally to enhance the function of the cerato-hyoid muscle [45], the contraction of which extends the dewlap. In other vertebrate species, androgen receptors occur in sexually dimorphic muscles that participate in male sexual behavior, such as those required for penile function in the rat [24] and for amplexic clamping or courtship vocalizations in the frog [14,17]. Although under investigation, the distribution of androgen receptor in the dewlap system in A. carolinensis is currently unknown.

Although the present results suggest that 5α-reductase activity facilitates the action of T in stimulating male courtship behavior in the green anole, many questions remain, such as precisely how and where DHT acts to facilitate the actions of T. Although both T and DHT can bind to the same receptor [61], they could differentially regulate transcription by altering the structure of the steroid receptor complex [66]. Alternatively, the brain of A. carolinensis might contain multiple isoforms of the androgen receptor, which differ in their affinities for T and DHT. The existence of multiple androgen receptor isoforms is not unusual and has been observed in tissues from frog [17], mouse [60], and human [18]. Also, the present data are consistent with the possibility that 5α-reduced metabolites of other steroids are important. For example, 5α-reductase acts on progesterone to convert it into dihydroprogesterone [6]. Studies of laboratory rats [63,64], as well as the lizard species Chelys dophorus inornatus [28–31], C. uniparens [21], and A. carolinensis [65] indicate that progesterone can act alone or synergize with androgens to elicit male sexual behaviors. It has yet to be determined whether progesterone is metabolized by 5α-reductase prior to mediating anole sexual behaviors. The present results indicate that this possibility merits investigation.

In conclusion, the results of the current study indicate that 5α-reductase activity is necessary for courtship behaviors in A. carolinensis. However, based on these results and on those of previous studies (see above), T seems to be the primary regulator of male sexual behaviors in this species, and 5α-reduced metabolites play only a supportive, albeit necessary, role. Perhaps the anole represents a more ancestral condition to birds and mammals, in which the mechanisms are present to utilize T as a prohormone (e.g., steroid metabolizing enzymes and estrogen receptors), but the role of T-metabolites as direct mediators of behavior did not evolve until later.

Acknowledgments

We thank Erin O’Bryant for help with injections, Camilla Peabody for technical assistance, and Ed Rufle for measuring the renal sex segments in Experiment 2. FCE 28260 (PNU 156765) was a generous gift of Pharmacia and Upjohn. The present study was supported by NSF grant IBN-973074 to J.W. and NIH postdoctoral NRSA MH12361 to G.R.

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