Testosterone secretion varies in a sex- and stage-specific manner: insights on the regulation of competitive traits from a sex-role reversed species

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Highlights

- Hormones in role reversed species can reveal the influence of parental vs gonadal sex
- In jacanas, females face stronger intra-sexual competition, and males care for offspring
- We examine covariation between testosterone (T) and mating competition
- Circulating T is similar in females and incubating males, but higher in copulating males
- T levels co-vary with different competitive traits, depending on sex and breeding stage.

Abstract
Testosterone (T) mediates a variety of traits that function in competition for mates, including territorial aggression, ornaments, armaments, and gametogenesis. The link between T and mating competition has been studied mainly in males, but is less understood in females, who also face selection pressures to compete for mates. Sex-role reversed species, in which females are the more competitive sex, provide a unique perspective on the role of T in promoting competitive traits. We examined whether T co-varies with competitive traits in sex-role reversed Northern Jacanas (*Jacana spinosa*) during breeding, when females are fertile and males are either seeking copulations or conducting parental care. We collected data on baseline levels of T in circulation in both sexes, and measured a suite of behavioral and morphological traits putatively involved in mating competition. Contrary to nearly all prior work on sex-role reversal, we did not find a significant main effect of sex on T secretion. Instead, females and incubating males had similar levels of T secretion, which were lower than those observed in copulating males. Nevertheless, females had higher expression of a suite of competitive traits, and their T was higher than most tropical birds. In females, T positively correlated with wing spur size, but was uncorrelated with other competitive traits, including aggression. In males, T correlated with facial shield size and body mass, and among copulating males, T also predicted testes mass. These findings suggest that the mechanisms regulating competitive traits vary with reproductive role in ways that are unique from gonadal sex.

Keywords: testosterone, sex-role reversal, intra-sexual selection, female competition, male parental care

Introduction

For males of many vertebrate species, testosterone (T) has been associated with phenotypic traits that lead to successful competition over mates. These traits include aggressive behavior (Wingfield et al., 1990), weaponry (Malo et al., 2009), body size (Cox et al., 2009), ornamentation (McGlothlin et al., 2008), and testes size (Preston et al., 2012). Females also express many of these same traits, which likewise function in competition for mates and other breeding resources (Clutton-Brock, 2009; Hare and Simmons, 2018; Rosvall, 2013a; Tobias et al., 2012). Like males, females secrete T and have the physiological machinery to respond to T (Staub and De Beer, 1997). However, the relationship between T and mating competition in females has found more equivocal support

(Goymann and Wingfield, 2014; Rosvall et al., in press, Cain and Ketterson 2011), suggesting that T may influence trait expression differently in males and females. This can be explained in part by sex differences in the selective pressures that drive endocrine mechanisms of behavior (Wingfield et al. 1990). For instance, the relative importance of aggression versus parental care in males and females may shape both sexual dimorphism in T, as well behavioral sensitivity to T (Lynn, 2008; Rosvall, 2013b).

Levels of T in circulation are typically elevated at the beginning of the breeding season, when competition for mating opportunities is high, and then T levels decline as behavioral efforts shift to parental care (Wingfield et al. 1990). This cross-stage shift in T production has been well demonstrated in males (Hirschenhauser and Oliveira, 2006), and is potentially more dramatic in females (DeVries et al., 2012; George and Rosvall, 2018; Jawor et al., 2007). Cross-stage shifts may also alter correlations between T and sexually selected traits, such that these traits are strongly integrated with T during competition for mates, but more independent from T during periods of parental care (Ketterson et al., 2009; Lipshutz et al., 2019a). An open question is how reproductive roles impose sex-specific selection pressures on the phenotypic covariation between T and competitive traits, especially if these traits are under stronger selection in a particular breeding stage and/or for a particular sex (Hämäläinen, 2018).

In sex-role reversed species, females face stronger selection to compete for mates, and males predominantly care for offspring (Emlen and Oring, 1977), providing a unique opportunity to disentangle reproductive role from gonadal sex. Whereas sex-role reversed females are freed from the constraints of parental care, selective pressures to care for offspring may limit levels of T in males. The hypothesis that sex-role reversed females have higher T than males has intrigued behavioral ecologists for decades. Whereas early studies in sex-role reversed phalaropes found higher T in ovarian than testicular tissue (Höhn, 1970; Höhn and Cheng, 1967), most studies have found that the direction and magnitude of sex differences in T is typically similar between role-reversed species and species with traditional sex roles (Wilson's phalaropes - Fivizzani et al., 1986; red-necked phalarope - Gratto-Trevor et al., 1990; spotted sandpiper - Rissman and Wingfield, 1984, African black coucal - Goymann et al., 2004; but see barred button-quails Muck and Goymann 2011). Approaching sex-role reversed species from a hormonal perspective can shed new light on the regulation of competitive traits, which may vary based on gonadal sex and/or reproductive role. Of the few studies that have examined phenotypic co-variation with T in sex-role reversed species, one found that T positively correlated with plumage coloration and body condition in females only (Muck and Goymann, 2011), and the other found that ornaments were testosterone-dependent in both sexes (Eens et al., 2000). Neither of these studies examined the influence of breeding stage on phenotypic covariation, which is important for considering how temporally variable selection pressures (i.e. mating competition vs. parental care) shape differences both between and within the sexes.

One of the most well-known examples of sex-role reversal is the jacana (family Jacanidae). Although both sexes aggressively compete for territories on which to breed, female jacanas face more intense sexual selection, have higher potential reproductive rates, and have female-biased operational sex ratios (Emlen and Wrege, 2004a). Female jacanas also have larger secondary sexual traits, including weaponry, body mass, ornamentation, and behavioral dominance (Emlen and Wrege, 2004a; Lipshutz, 2017; Stephens, 1984). Males provide nearly all parental care, including incubation and foraging with chicks (Emlen and Wrege, 2004b; Jenni and Collier, 1972). Although jacanas are a classic example of female-biased dimorphism in behavior and morphology (Emlen and Oring, 1977), there is currently no published work on the physiological mechanisms that regulate the expression of these traits. Here, we quantified circulating T levels in free-living, sex-role reversed jacanas to evaluate the hypotheses that 1) levels of T track gonadal sex 2) reproductive roles influence T secretion in males, and 3) covariation between T and competitive traits varies in a sex- and stage- specific manner. Sex-role reversed females should be unconstrained by parental care, and if they have higher T than males, this would suggest that T levels track reproductive roles more so than gonadal sex. We further predict that males incubating eggs should have lower T than males seeking copulations. We also

predict T will be more strongly linked with competitive traits in females and copulating males, but that T should be more independent from these traits in incubating males.

Materials and Methods

Subjects

Northern Jacanas (*Jacana spinosa*) are medium-sized tropical shorebirds found throughout Central America, from Mexico to Panama. They breed asynchronously and year-round, although breeding increases during the rainy season in Panama from roughly May to October. We conducted fieldwork from 4 June to 9 July 2018 in La Barqueta, Chiriqui (8.207N, 82.579W).

Aggression assays, plasma collection, and morphological measurements

Prior to each aggression assay, we observed each individual for several days to ensure that it was pair-bonded (e.g. foraging with mate) and territorial (e.g. actively defending territory from intruding floaters). Territorial residents behave distinctly from floaters, who do not breed nor defend territories (Emlen and Wrege, 2004a). We also determined breeding status based on whether males were copulating or incubating a nest. We did not color-band individuals as in previous studies (Lipshutz et al., 2019b), but territory holders have high site fidelity (Emlen and Wrege, 2004a), and the agricultural land where we conducted this research has small ponds and canals where territorial boundaries are distinct. We simulated territorial intrusion in males (n = 12) and females (n = 10) using female taxidermic mounts and conspecific vocalizations following Lipshutz (2017). Briefly, we set up a camouflage blind and placed the mount and speaker in the center of a female's territory, and used a random combination from four acoustic stimuli and 4 taxidermic mounts. We measured a suite of aggressive behaviors, including average distance from the mount, hover flights, wing spreads, flyovers, and vocalizations, described previously in an ethogram for jacanas (Lipshutz, 2017). Because circulating T levels have the potential to change within 10 minutes of experimentally initiating aggression (Wingfield and Wada, 1989; but see Goymann et al., 2019), we shortened the assay to 5 minutes.

Following the assay, 9 individuals were captured and shortly thereafter euthanized for another study (i.e. "immediate collection"). We were unable to collect 13 individuals within five minutes post-assay, and so, we returned 5-8 days later to collect these individuals ("i.e. delayed collection"). To obtain unprovoked levels of T, we collected individuals using an air rifle, followed by an anaesthetic overdose of isoflurane and decapitation to collect trunk blood (average time from assay start to euthanasia = 9 min 20 sec ± 1 min 35 sec for immediate collection individuals). We collected whole blood into heparinized BD Microtainers (product #365965) and stored on an ice pack until we separated plasma by centrifuging for 10 minutes at 10,000 rpm. We stored plasma at -20°C for later testosterone assays. For delayed collections, we ensured that breeding stage did not change; for instance, if a male was incubating on the day of the assay, we confirmed that he was still incubating on the day of collection. For each sex, we balanced immediate and delayed collection sample sizes. We confirmed sex and breeding status by examining whether females had hierarchical follicles, and whether males had brood patches. We determined that 7 males were incubating, 5 were copulating, and all 10 females were fertile, with hierarchical follicles.

Morphological measurements

Postmortem, we measured several traits putatively involved in mating competition in jacanas: wing spur length, body mass, facial shield length, and gonad mass. In a congener, the Wattled Jacana, territorial resident status was associated with larger wing spurs, facial shields, and body mass for both sexes, and only territory holders can breed (Emlen and Wrege, 2004a). These traits are much smaller in floaters, who do not obtain territories or reproduce. We assume that these traits similarly relate to intra-sexual competition in Northern Jacanas, although we have not tested this explicitly. For both species, wing spurs, facial shields, and body mass are

significantly higher in adult female jacanas compared to males (Emlen and Wrege, 2004a; Lipshutz, 2017), but these traits are small in juveniles of both sexes (Lipshutz, personal observation). We measured body mass with a digital scale (0.01g), and we measured wing spur length (from base center to tip, 0.1 mm) with calipers. Wing spurs are sharp, yellow keratinous sheaths over metacarpal bone growths that jacanas use as weapons and display during aggressive posturing. In the Wattled Jacana, wing spurs length positively correlated with age in males but not females, perhaps because they were worn down by abrasion from fighting (Emlen and Wrege, 2004a). However, we did not observe any worn down wing spurs in male or female jacanas. In Northern Jacanas, facial shields are yellow, fleshy, and extend from the upper mandible to the forehead. We measured facial shield length (from right nare to top right lobe, 0.1 mm) with calipers. We also measured gonad mass with a digital gem scale (0.1oz). Gonad mass has clear connections to mating competition because jacanas are polyandrous, and males that are simultaneously mated with a single female compete to fertilize her eggs (Emlen et al., 1989). Testes size is associated with T in many avian species (Garamszegi et al., 2005), and testes size is correlated with sperm length in shorebirds (Johnson and Briskie, 1999).

Testosterone enzyme immunoassay

We extracted steroids from plasma samples using diethyl ether (3x extractions) and reconstituted in 250µL assay buffer. We measured testosterone using a High Sensitivity Testosterone ELISA kit (Enzo #ADI-900-176, Farmingdale, NY, USA) following methods described in George and Rosvall (2018). We confirmed assay parallelism by comparing concentrations from a standard curve made by kit standards to a displacement curve made by dilutions of a copulating male jacana (R_2 = 96.3%). We ran all samples in duplicate. We initially used 40µL plasma from females, and 20µL from males. Samples from three females and four copulating males initially showed less than 20% maximum binding, and two males showed greater than 80% maximum binding, so we re-ran them using 10µL plasma and 40 uL of plasma, respectively, to obtain values in the most sensitive part of the curve. We calculated T concentration by comparing sample absorbance with the absorbance of the assay's standard curve (Gen5 curve-fitting software, Biotek EPOCH plate reader, Winooski, VT, USA). Intra-assay CV was 4.44% and inter-plate CV was 5.94%.

Statistical analysis

We conducted all statistics in R version 3.3.2 (R-Core-Team, 2015). We examined normality using a Shapiro-Wilk normality test and examined outliers using a Grubbs test in the R package outliers. We normalized T using a log scale transformation. There were no differences in testosterone levels between individuals that were collected immediately versus delayed ($t = -0.0081$, $df = 19.87$, p-value = 0.99), so we combined these groups for analysis. To compare T, morphology, and aggression between the sexes at different breeding stages, we used a one-way ANOVA, followed by a Tukey post hoc test. Comparisons between sexes or male breeding stages were made using Student's t tests or Wilcoxon tests, depending on normality. To assess the degree of co-variation among T, competitive, and reproductive traits, we used Spearman's correlations.

We also summarized the 5 aggressive behaviors with a principal component analysis (PCA) using the prcomp function. We retained 1 PC with an eigenvalue > 1 (hereafter 'Aggression PC1'), which explained 64.1% of the variation in the aggressive behaviors (Table 1). Distance loaded negatively onto Aggression PC1, and hover flights, wing spreads, flyovers, and vocalizations loaded positively, such that a more positive PC1 reflects a more aggressive response.

Table 1. Loadings for Principal Component Analysis of aggressive behavior

Vocal Parameter	PC1
Eigenvalue	1.79
Proportion of variance	64.14%
Distance to mount	-0.41
Hover Flights	0.43
Wing Spreads	0.46
Flyovers	0.46
Vocalizations	0.47

Results

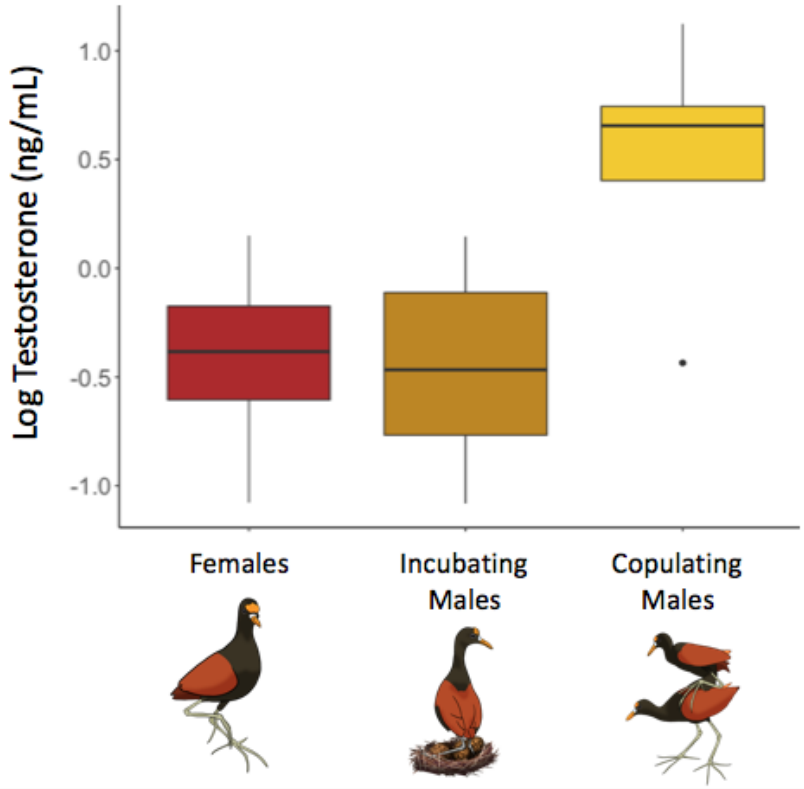

Figure 1. Circulating testosterone levels in female (n = 10) and male jacanas at different breeding stages, incubation (n = 7) and incubation (n = 5). The thick horizontal line represents median values of logT, outlined by the first and third quartiles, with vertical lines representing minimum and maximum values. The outlier copulating male was growing in feathers over his brood patch.

Circulating testosterone varies by breeding stage

When all males were combined, male and female T were not significantly different ($t = -1.58$, $df = 17.47$, $p = 0.13$). When considering breeding stage however, the groups were significantly different ($F_{2,19} = 8.2$, $p = 0.0027$; Fig. 1). Copulating males had significantly higher T levels than incubating males (5.26 ± 2.2 vs 0.54 ± 0.18 ng/mL, Tukey: $p = 0.005$). One copulating male had T similar to the average T levels of incubating males, but was not an outlier among other copulating males ($p = 0.099$); upon collection he was observed to have new feathers growing in over his brood patch. Females had T levels

(0.51 ± 0.13 ng/mL) similar to incubating males and significantly lower than copulating males (Tukey: $p = 0.004$).

Competitive traits vary by sex and breeding stage

We confirmed female-biased dimorphism in traits putatively involved in competition for mates, as has been demonstrated in jacanas (Emlen and Wrege, 2004a; Lipshutz, 2017). Females had significantly longer wing spurs ($t = 10.23$, $df = 20.49$, $p < 0.0001$), larger body mass ($t = 21.89$, $df = 18.54$, $p < 0.0001$), and longer facial shields ($t = 9.69$, $df = 19.0$, $p < 0.0001$) than males. Between males in different breeding stages, incubating males had significantly lower testis mass than copulating males ($t = -2.57$, $df = 8$, $p = 0.033$), and a trend towards longer wing spurs ($t = 2.08$, $df = 5.15$, $p = 0.091$).

Between males and females, there was no significant difference in Aggression PC1 ($t = 1.61$, $df = 16$, $p = 0.13$). However, when considering male breeding stage, groups differed significantly in Aggression PC1 ($F_{2,19} = 3.89$, $p = 0.038$). Incubating males were significantly more aggressive than females (Tukey: $p = 0.049$), and there was a trend towards higher aggression in incubating males than copulating males (Tukey: $p = 0.089$).

Phenotypic integration with testosterone *varies by sex and breeding stage*

In females, T correlated positively with wing spur length, but not with other putatively competitive or reproductive traits (Table 2). Among all males, T positively correlated with body mass and facial shield length. However, when males were parsed by breeding stage, T was positively correlated with testes mass among copulating males, but uncorrelated with any trait among incubating males. T was not correlated with Aggression PC1, regardless of sex or breeding stage.

Table 2. Spearman correlations (r_s) of circulating testosterone with competitive traits for females, males combined, and incubating and copulating males. Significant relationships ($p \leq 0.05$) are bold.

	Females	All Males	Incubating Males	Copulating Males
Wing Spur Length	**$r_s = 0.83$** **$p = 0.0031$**	$r_s = -0.47$ $p = 0.13$	$r_s = -0.07$ $p = 0.91$	$r_s = -0.70$ $p = 0.23$
Body Mass	$r_s = 0.09$ $p = 0.80$	**$r_s = 0.70$** **$p = 0.014$**	$r_s = 0.29$ $p = 0.56$	$r_s = 0.70$ $p = 0.23$
Facial Shield Length	$r_s = -0.21$ $p = 0.57$	**$r_s = 0.69$** **$p = 0.01$**	$r_s = 0.57$ $p = 0.2$	$r_s = 0.4$ $p = 0.52$
Gonad Mass	$r_s = 0.25$ $p = 0.55$	$r_s = 0.49$ $p = 0.13$	$r_s = -0.64$ $p = 0.17$	**$r_s = 0.97$** **$p = 0.0048$**
Aggression PC1	$r_s = -0.061$ $p = 0.87$	$r_s = 0.30$ $p = 0.35$	$r_s = -0.29$ $p = 0.56$	$r_s = 0.45$ $p = 0.50$

Discussion

In wild female and male jacanas, we found that differences in T secretion were contingent on male breeding stage, rather than sex alone. Circulating T levels in females were similar to levels observed in males conducting parental care. This contrasts with nearly all prior work on sex-role reversed species, for which females have lower levels of T in circulation than males, despite being the more competitive sex. Copulating males had higher T than incubating males, suggesting that T and parental care may constrain one another. We also found that phenotypic covariation of T with traits putatively involved in mating competition differed between females and males, and in males, these patterns varied with breeding stage.

In male jacanas, T differs by breeding stage, suggesting that levels of T are shaped by shifting selection pressures from parental effort to mating effort. This pattern is similar to studies in other sex-role reversed species like spotted sandpipers (*Actitis macularia*), in which T was similarly low for both

females and nesting males, and higher in non-nesting males (Rissman and Wingfield, 1984). These patterns have also been found more broadly in non-sex-role reversed species with male parental care (Wingfield et al., 1990). Experimental evidence that exogenous T reduces male parental care in spotted sandpipers (Oring et al., 1989) as well as socially monogamous songbirds (Goymann and Davila, 2017; Ketterson et al., 1992; Van Roo, 2004; but see Lynn, 2008) indicates that high T can inhibit male parental behavior regardless of whether males are the more competitive sex. This idea was supported by a meta-analysis across vertebrates, finding that male parental care predicted T but mating system did not (Hirschenhauser and Oliveira, 2006), and another meta-analysis in humans, finding that fathers have lower T than men without children (Grebe et al., 2019).

Given the constraints of egg production and parental care typically imposed on females, T levels ought to be less correlated with suites of integrated traits in females than in males (Ketterson et al., 2009). For sex-role reversed species like jacanas, in which males conduct all parental care, we expected this relationship to reverse between females and males. However, we did not find strong phenotypic covariation with T in females, nor in males divided into breeding stage. In females, T was strongly and positively correlated with wing spur length, a weapon used to fight over territories and mates. Studies in sex-role reversed barred buttonquail females (*Turnix suscitator*) found that female, but not male levels of T were positively correlated with body condition and the size and blackness of the melanin throat patch (Muck and Goymann, 2011). When we combined males from both breeding stages, we found that T correlated positively with both body mass and facial shield length. These correlations appear to be driven by stage-related variation in all of these traits, rather than dose-dependent relationships between T and individual differences in competitive traits. In particular, when we divided males by breeding stage, only testes mass was correlated with T in copulating males, a pattern found repeatedly in males of many avian species (Garamszegi et al., 2005). T did not correlate with aggression for either sex, regardless of breeding stage. This is not unexpected, given that aggression and baseline levels of T do not correlate for many species (Kempenaers et al., 2008; Williams, 2008), although it is possible that such correlations would emerge during certain situations (e.g. during territorial establishment) or when T secretion is at its physiological maximum (e.g. endogenous or exogenous activation of the HPG axis). Our findings nevertheless reveal sex- and stage- specific variation between T and different competitive traits, suggesting that both gonadal sex and reproductive roles independently feed back to influence T secretion.

These sex- and stage-specific patterns further suggest that other aspects of the androgenic signaling system must also influence the expression of competitive phentoypes; afterall, female jacanas have T levels that are on par with incubating males, but they express an exaggerated suite of competitive traits. Circulating T is only one component of the androgen signaling system, and regulatory mechanisms of competitive traits in both sexes could also depend on T-responsiveness in neural and peripheral tissues, including tissue-specific variation in T production, metabolite conversion, and androgen or estrogen receptors (Ball and Balthazart, 2019; Fuxjager and Schuppe, 2018; Schmidt et al., 2008; Soma, 2006; Staub and De Beer, 1997). Such tissue-specific variability may regulate phenotypic traits independently of circulating sex steroids (Bentz et al., 2019; Horton et al., 2014; Lipshutz et al., 2019a; Rosvall et al., 2012), but there is some evidence that tissue-specific versus systemic regulation of sex steroid mediated traits may be more prevalent for groups in which T levels are depressed (Demas et al., 2007; Rosvall, 2013b). Sex differences in androgenic signaling in the brain have been found in several sex-role reversed species; female black coucals and barred button quails had higher mRNA expression of androgen receptors (AR) in neural regions implicated in the control of aggressive and sexual behavior (Voigt, 2016; Voigt and Goymann, 2007). An exciting next step is to use the unique jacana system to explore how gonadal sex and parental role influence tissue-level regulation of competitive phenotypes.

During the rainy season in Panama, territorial female jacanas breed simultaneously with multiple males in their harems, and males copulate and incubate asynchronously. All females in this study displayed clear signs of fertility (i.e. hierarchical follicles), whereas males had large

testes only when copulating, but small testes when incubating. In this sense, the continuously fertile gonadal state of female jacanas is similar to other species for which males maintain large, fertile testes throughout female incubation. Likewise, the cycling gonadal size of male jacanas from copulation to incubation is analogous to other species for which female ovaries return to a non-fertile state during incubation (Williams, 2012). In sum – stage-related fluctuations in gonadal state are also reversed between female and male jacanas, in contrast to our finding that females did not have higher T than males. This finding could relate to the fact that jacanas are tropical, and some maintain breeding territories year-round (Emlen and Wrege, 2004a). Tropical birds typically have lower levels of circulating T compared with temperate species, due to longer and more asynchronous breeding (Garamszegi et al., 2008; Goymann et al., 2004a; Hau et al., 2010). However, when we compared Northern Jacana to other tropical species with year-round territoriality (T < 2.0 ng/mL, Goymann et al. 2004a), we found that females and incubating males had similar T to males of these other species, and copulating males had much higher T. In contrast to tropical jacanas, the majority of sex-role reversed avian species studied to date are temperate and migratory. The essentially continual state of female jacana fertility, paired with sex-role reversal, should generate stronger or more uniform selection on competitive traits, which are advantageous during most the year and do not experience counter-selection in relation to wintering or migratory states. Our examination of T's relationship to competitive traits brings us one step closer to understanding how gonadal sex and reproductive roles influence mechanisms of behavior.

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