

Females competing to reproduce: Dominance matters but testosterone may not

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Abstract

The associations among aggression, testosterone (T), and reproductive success have been well studied, particularly in male birds. In many species, males challenged with simulated or real territorial intrusions increase T and levels of aggression, outcomes linked to higher dominance status and greater reproductive success. For females, the patterns are less clear. Females behave aggressively towards one another, and in some species, females respond to a social challenge with increases in T, but in other species they do not. Prior work on female dark-eyed juncos (*Junco hyemalis*) had shown that experimental elevation of T increases social status and intrasexual aggression. Here, we conducted two experiments designed to answer three questions: Are endogenous concentrations of T associated with dominance status in captive female juncos? Does dominance status influence readiness to breed in female juncos? And do captive females increase T in response to a challenge? In the first experiment, we introduced two females to a breeding aviary, allowed them to form a dominance relationship and then introduced a male. We found that dominant females were more likely to breed than subordinates, but that dominance status was not predicted by circulating T. In the second experiment, we allowed a resident male and female to establish ownership of a breeding aviary (territory) then introduced a second, intruder female. We found that resident females were aggressive towards and dominant over intruders, but T did not increase during aggressive interactions. We suggest that during the breeding season, intrasexual aggression between females may influence reproductive success, but not be dependent upon fluctuations in T. Selection may have favored independence of aggression from T because high concentrations of T could interfere with normal ovulation or produce detrimental maternal effects.

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In male vertebrates, the associations among testosterone (T), aggression, and dominance status have been well studied, particularly in birds (Canoine and Gwinner, 2002; Hau et al., 2000; Schlinger et al., 2001; Silverin et al., 2004; Van Duyse et al., 2003; Wingfield, 1994; Wingfield et al., 2000). Among many seasonally breeding birds, male T increases from near undetectable non-breeding concentrations to much higher concentrations in the early breeding season during territory acquisition and pair formation (Hegner and Wingfield, 1987; Ketterson et al., 1991; Van Duyse et al., 2003; Wingfield, 1994; Wingfield et al., 1990). When a male is socially challenged during the breeding season, an additional transitory increase in circulating T may follow, and this increase in T is often

accompanied by elevated aggression towards challengers (the “challenge hypothesis”, Geslin et al., 2004; Johnsen, 1998; Smith et al., 2005; Wingfield, 1994; Wingfield et al., 1990; but see Foerster and Kempenaers, 2005; Hau et al., 2000, 2004; Moore et al., 2004; Van Duyse et al., 2004; Wiley and Goldizen, 2003; Wingfield et al., 1992 for instances where T does not increase, primarily in tropical species). Among males, T-mediated increases in aggressive behavior and dominance status may enhance access to breeding territories, high quality breeding resources, and mates (reviewed in Ketterson and Nolan, 1999; Wingfield et al., 2000).

Female birds also behave aggressively towards one another (Jawor et al., 2004; Sandell, 1998; Slagsvold et al., 1999; Whittingham and Schwabl, 2002). Females are believed to compete for access to resources (territories, nest sites), males of high quality or males that might provide access to high quality

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resources, including paternal care (Creighton, 2001; Elekonich, 2000; Elekonich and Wingfield, 2000; Emlen and Wrege, 2004; Karvonen et al., 2000; Kim and Zuk, 2000; Owens et al., 1994; Slagsvold and Lifjeld, 1994). Additionally, females behave aggressively towards one another to protect nests sites and eggs/young (Jawor et al., 2004; Whittingham and Schwabl, 2002) and to deter their mates from acquiring secondary females (Arcese, 1989; Rätti et al., 1994; Sandell, 1998; Sandell and Smith, 1997; Slagsvold, 1993; Slagsvold et al., 1999). Despite the importance of female–female aggression in birds, the role of hormones in aggression, including T, is not as well understood as it is for males (see Elekonich and Wingfield, 2000; Hau et al., 2000; Ketterson et al., 2005; Langmore et al., 2002).

Generally, among seasonally breeding birds, endogenous T is lower in females than in males, and varies over the course of the year with higher concentrations observed during the breeding season (Clotfelter et al., 2004; Elekonich and Wingfield, 2000; Hegner and Wingfield, 1987; Ketterson et al., 2001, 2005). A small number of studies have investigated whether females elevate T in response to social instability and the findings have been mixed. Some studies have found that females in the midst of aggressive interactions increase circulating T (Hegner and Wingfield, 1987; Langmore et al., 2002; Mazuc et al., 2003; Smith et al., 2005), while others have not (Elekonich and Wingfield, 2000; Hau et al., 2004; Schwabl et al., 1988). In this study, we explored the association between, dominance, reproduction, and T in a population of captive female dark-eyed juncos (*Junco hyemalis*). Female juncos exhibit high plasma concentrations of T in the early spring (Ketterson et al., 2001, 2005), and females with experimentally increased T display more aggression than controls when presented with an intruder (Zysling et al., in review). However, the influence of naturally occurring concentrations of T on dominance and aggression in females has not been explored. In the first experiment, we allowed females to establish dominance relationships prior to the introduction of a male to the breeding aviary; in the second experiment, we allowed a female to establish residency in the breeding aviary and then introduced a second female as an intruder. In both cases, we compared dominance status to circulating T, male courtship, and to whether or not the females bred as measured by building nests and laying eggs. We predicted that females who became socially dominant would have higher naturally occurring T concentrations than subordinates either before or after they interacted, or both. We also predicted that socially dominant females would have a reproductive advantage over their subordinate counterparts.

Methods

General methods: daily care and housing

Studies were conducted in 2003 (experiment 1) and 2004 (experiment 2) from May to August at Kent Farm Bird Observatory, Indiana University, Bloomington, Indiana, USA. Prior to experiments, birds were housed in same-sex flocks (one male flock, two separate female flocks) in large aviaries. Females were housed in one of two indoor aviaries (6.4 m × 3.2 m × 2.4 m and 5.7 m × 5.4 m × 2.4 m) on a light schedule matching natural L:D cycles; males were housed in a large outdoor aviary (18.3 m × 4.6 m × 2.4 m). During experiments, both 1 and 2, trios of birds (2 females with 1 male) were housed in smaller

indoor and outdoor aviaries (2.6 m × 2.1 m × 2.4 m indoor, 2.4 m × 2.4 m × 2.4 m outdoor). For both experiments, the two females making up each dyad originated from separate flocks. Between experiments 1 and 2, which were conducted a year apart, female flocks were rearranged in order to ensure that females making up the dyads had not been housed together for at least 8 months. All birds received food (millet–sunflower seed mixture supplemented with a high-protein mixture of carrots, dried commercial dog food, and eggs) and water with vitamins *ad libitum*. All birds were originally captured at Mountain Lake Biological Station, Virginia, USA, and had been in captivity for at least 1 year. All work was done with permission from the Indiana University Bloomington Animal Care and Use Committee (study number 03-115) and the United States Fish and Wildlife Services (Special Purposes permit MB093279-0) and procedures were in keeping with the guidelines of the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Female intrasexual aggression, assessing aggressive behavior, and dominance

In both experiments, we determined dominance status by observing the behavior displayed by females based on descriptions in Balph (1977); aggressive displays included bill gaping, piloerection, displacements from perch sites, physical attacks, and chasing. The female that moved away following an interaction was considered the loser of that interaction. The female who won the majority of interactions observed was considered the dominant female. Dominance relationships in juncos, once determined, are stable over time and initial dominance interactions are highly predictable of future dominance relationships (Cristol et al., 1990). During experiment 1, dominance status of a given female was determined by independent observers performing 30 min observations on 2 consecutive days. In experiment 2, we quantified the frequency of aggressive interactions initiated by the resident female over the first 30 min following the introduction of the intruder.

Male behavior, courtship, and intersexual aggression

In both experiments, we determined which female the male preferred by noting which female he directed courtship towards. Male courtship includes soft short-range song, erected body feathers, tail-spreading, and the offering of nesting material (Nolan et al., 2002). In both experiments, males typically courted both females at first, but soon focused their courtship on a single female that we then considered to be preferred. Male preference of a particular female was based on two 30 min observations performed on 2 consecutive days by independent observers.

Experiment 1

In the first experiment, two females matched for weight ($\pm 5\%$ for mass) and wing chord length (± 2 mm), were placed simultaneously into breeding aviaries (9 dyads to inside aviaries and 13 dyads to outside aviaries for 22 total female dyads), and allowed to establish a dominance relationship prior to male introduction. Exact ages of birds varied, but females were matched for approximate age by combining older adults with older adults, yearlings with yearlings. Immediately before their introduction, a blood sample was collected from both females for later analysis of T. Blood samples were collected between 0800 and 1100; the average time required to capture and begin bleeding females was less than 3 min.

Following their introduction into the aviary, the females were observed for 30 min and their dominance status determined as already described. After a 48 h acclimation period, and additional observations to determine dominance status, we introduced a male into the breeding aviary, and for 30 min observed the behavior of all three birds. Trios stayed in breeding aviaries for up to 2 weeks and were checked daily to determine whether breeding had commenced. We determined which female was building the nest by observation and made daily checks for eggs. If neither female had built a nest within 2 weeks, experimental groups were dismantled and the birds were placed back in unisex flocks. Dyads were not more likely to initiate reproduction in the inside aviaries than the outside aviaries (8 of 13, 61% inside, 7 of 9, 77% outside, $\chi^2 = 0.64$, $df = 1$, $P = 0.42$), and data were combined in analyses below.

Experiment 2

To begin the resident–intruder experimental trials, we placed a male and a female in a breeding aviary (4 inside, 26 outside) and allowed the female to establish herself as the resident female. Female juncos rarely hold territories independently of males (Nolan et al., 2002) and we placed a male in the aviary to make the situation more natural and to promote reproduction. On the same day that the pair was established, we placed a second female, destined to be the intruder, in a cage by herself. As in the first experiment, females were matched for size and general age. We collected blood samples for later hormonal analysis from each female prior to the resident female's introduction to the male and the intruder female's placement in isolation (hereafter baseline 1). After 48 h of acclimation (resident female to the male, intruder female to isolation), we again collected blood from both females (hereafter baseline 2). Twenty-four hours after the second blood sample was collected, we introduced the intruder female into the breeding aviary with the resident female and observed interactions between the birds for 30 min. Both females were then immediately captured (capture took on average 2 min) and a final blood sample was collected (hereafter post-challenge). Both females and the male remained in the breeding aviary for 2 weeks while we monitored breeding activity. If no breeding was observed, groups were dismantled and birds were placed back in unisex flocks. Dyads were not more likely to initiate reproduction in the inside aviaries than the outside aviaries (8 of 26, 31% outside, 1 of 4, 25%, inside, $\chi^2 = 0.055$, $df = 1$, $P = 0.81$), and data were combined in analyses below.

Hormone assays

Each time we bled the females, we collected 50–100 μ l of blood from the wing vein; the blood was centrifuged and the plasma fraction was drawn off and stored at -20°C until assayed. We determined T concentrations using an EIA kit (Assay Designs, Inc., #901-065) (described in Clotfelter et al., 2004). For the analysis of 2003 samples, approximately 2000 cpm of $\text{H}^3\text{-T}$ were added to allow calculation of recoveries after extraction (2 extractions with diethyl ether). Because recoveries tended to be quite uniform, in 2004, we simplified the process by omitting the recovery step: instead, we ran a small number of additional standards to which we added label to determine an average recovery for each plate. T concentrations were determined with a 4 parameter logistic curve-fitting program (Microplate Manager; Bio-Rad Laboratories, Inc.) and corrected for incomplete recoveries. Intraassay variation, which was calculated as the coefficient of variation of values obtained from standard samples of known concentration, ranged from 3.01 to 8.72%, interassay variation was 8.46%.

Statistical analyses

For all statistical analyses, we used SPSS 11.5 (2003 SPSS, Inc., Chicago, IL, USA). In both experiments, T concentrations were corrected for interassay variation and log transformed to allow the use of parametric statistics. In both experiments, we used a Bonferroni corrected critical value when performing multiple comparisons (Sokal and Rohlf, 1995). We report P levels with each statistical test, and we note whether a result loses significance following Bonferroni adjustment and the adjusted significance level.

In experiment 1, given the categorical nature of the behavioral data collected for each female (dominant/subordinate, courted by male/not courted by male, bred/did not breed), we used Chi-square tests to determine whether dominance status was independent of whether or not females bred and whether dominance status influenced male preference. We used independent samples t tests to determine whether initial T concentrations varied according to female dominance status, whether or not females were courted by the male, and whether or not females bred.

In experiment 2, we used Chi-squared tests to determine whether dominance status or residence status was associated, and whether either dominance status or residence status influenced a female's ability to breed or whether she was preferred by the male. To determine whether female dominance status, residence status, whether or not a female was courted, and whether a female bred or not influenced T concentrations, we used repeated measures ANOVA with each category serving as a between-subject factor. Last, we used Pearson correlations to determine if the rate of aggression shown by residents was influenced by

concentrations of T. All repeated measures ANOVAs failed tests of sphericity, and we report Greenhouse-Geisser F statistics and P values.

Results

Experiment 1

A total of 22 dyads consisting of 44 females were monitored, giving rise to 22 female dominants and 22 subordinates. Dominance relationships were established quickly (always in the initial observations), and were stable with no reversals in dominance either before or after the male was introduced into the aviary. In 15 of these 22 female dyads (68%), at least one female initiated reproduction; in one case, both females initiated reproduction. Dominance status had a significant impact on breeding attempts. Twelve of the 22 dominant females (54%) initiated reproduction, as compared to 3 of the 22 subordinate females (13%) ($\chi^2 = 8.19$, $df = 1$, $P = 0.004$). Females that were preferred were also more likely to initiate reproduction ($\chi^2 = 5.054$, $df = 1$, $P = 0.02$, Bonferroni correction critical P value for two comparisons is 0.025). Intrasexual dominance is not likely to be a proxy for male preference because males were not more likely to prefer dominant females over subordinate females ($\chi^2 = 0.111$, $df = 1$, $P = 0.73$).

Interestingly, in 4 of the 15 breeding attempts (26%), we observed active interference in addition to aggressive behavior. For example, in one case, the dominant female repeatedly destroyed the nest the subordinate female was attempting to build. On three occasions, we were able to identify intraspecific brood parasitism based on inordinately large clutches (6 or more eggs) or extreme differences in egg appearance (shell coloration and egg size). In two cases, both females attempted to incubate eggs and brood young (either sequentially or simultaneously), one of these cases had evidence of intraspecific brood parasitism while in the other it was not clear if this had occurred.

T concentrations did not differ significantly between females who were later judged to be dominant and females later judged to be subordinate (independent samples t test; $t = 0.77$, $df = 34$, $P = 0.44$). Mean T for females later judged to be dominant was $0.472 \text{ ng/ml} \pm 0.05 \text{ ng/ml}$ while mean T for females later judged subordinate was $0.419 \text{ ng/ml} \pm 0.03 \text{ ng/ml}$. When T concentrations were categorized according to whether or not females later bred or whether or not they were preferred by the male, again there was no difference in initial T based on these categorizations (t tests; P s > 0.75).

Experiment 2

Thirty resident–intruder experimental trials were performed, and in each case, one of the females became dominant to the other. Dominance relationships were clearly determined during the initial 30 min observations, and no reversals in dominance were detected during additional observations. Nine of the 60 females (15%) initiated reproduction, 6 of these were females who had full blood sample collection (see below). Resident females were more likely to be dominant to intruder females ($\chi^2 = 23.273$, $df = 1$, $P < 0.0001$), in one case, an intruder female gained dominance over the resident female during the initial

introduction. Resident females were more likely to initiate aggressive interactions than intruder females ($\chi^2 = 10.87$, $df = 1$, $P = 0.001$). Resident females were more likely to reproduce than intruder females, but not significantly so ($\chi^2 = 3.310$, $df = 1$, $P = 0.07$), in large part because the one intruder female who gained dominance over the resident female later initiated reproduction. However, as in the first experiment, dominant females were more likely to reproduce than subordinate females ($\chi^2 = 8.666$, $df = 1$, $P = 0.003$). Males were not more likely to court residents over intruders or dominants over subordinates ($P_s > 0.25$).

Unlike the first experiment, we did not observe females actively interfering with one another during reproductive attempts, nor was there any obvious evidence of brood parasitism. We observed one case in which both females incubated eggs, the case where the intruder female gained dominance over the resident and initiated a nest. In this case, the females incubated the eggs sequentially (one female alone on nest at all times).

Of the 60 females, 43 provided blood samples for each interval (first baseline, second baseline, post-challenge). Of these 43 females (23 residents, 20 intruders), 6 (13%) built nests and laid eggs. Repeated measures ANOVA revealed no changes in concentrations of T over time when females were categorized according to resident–intruder status ($F = 2.114$, $df = 2$, $P = 0.14$), whether the female was dominant or subordinate ($F = 2.96$, $df = 2$, $P = 0.07$), preferred by the male or not ($F = 2.081$, $df = 1$, $P = 0.14$), or able to breed or not ($F = 0.705$, $df = 2$, $P = 0.46$). Furthermore, repeated measures ANOVA revealed no difference in concentration of T in resident versus intruder females ($F = 1.749$, $df = 1$, $P = 0.19$, Fig. 1A), dominant versus subordinate females ($F = 0.511$, $df = 1$, $P = 0.48$, Fig. 1B), preferred versus non-preferred females ($F = 1.021$, $df = 1$, $P = 0.31$), or females who did or did not initiate reproduction ($F = 1.073$, $df = 1$, $P = 0.30$).

Resident females displayed higher rates of aggression than intruder females (independent samples t test, $t = 3.96$, $df = 41$, $P < 0.0001$). However, when we examined whether the rate at which resident females initiated aggressive encounters during the 30 min intruder introduction was related to levels of T at any of the three time points (baselines 1 and 2, post-challenge), we found no correlations at either baseline or at post-challenge (Pearson correlation, all $P_s > 0.50$). Additionally, there was no correlation between rates of aggression displayed, irrespective of residence status, and change in T (baseline 1 to post-challenge) ($r = 0.08$, $P = 0.57$, $n = 43$). Last, we focused on female T in a subset of dyads in which the number of interactions between birds was quite high (15 to 32 interactions over 30 min), and we found no difference in the change in T between birds ultimately judged dominant or subordinate (paired samples t test; $t = 0.72$, $df = 10$, $P = 0.48$).

Discussion

In two experiments, one in which females of the same resident status competed for dominance status, and a second in which an intruding female was introduced to a resident female, we found that when two female juncos are housed with one

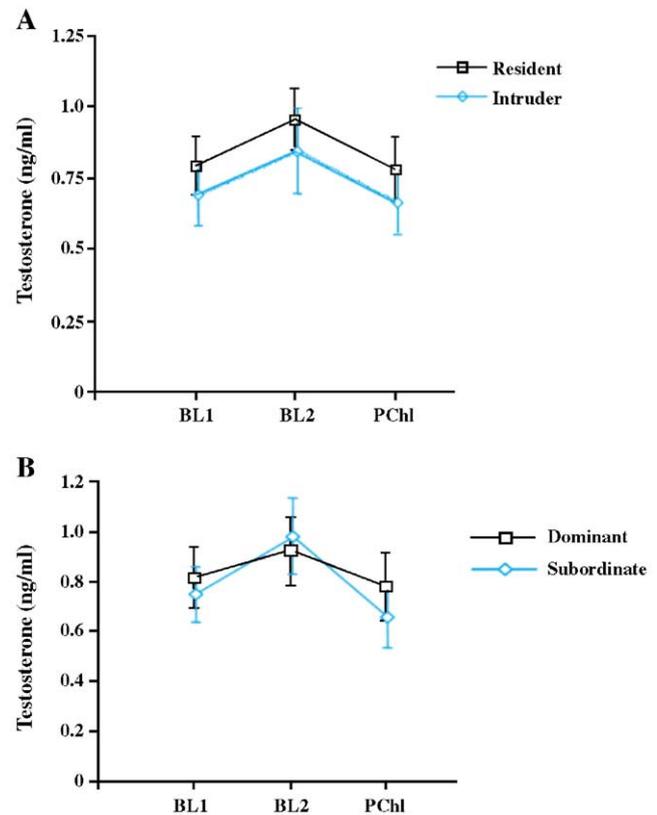


Fig. 1. (A) Testosterone concentrations for resident and intruder female dark-eyed juncos at baseline 1 (BL1), baseline 2 (BL2), and post-challenge (PChl) during experiment 2 (2004). (B) Testosterone concentrations for dominant and subordinate female dark-eyed juncos at baseline 1 (BL1), baseline 2 (BL2), and post-challenge (PChl) during experiment 2 (2004). Error bars represent ± 1 SEM.

male, the ability of one female to dominate the other is a significant predictor of which of the two females will initiate reproduction. In some cases, neither female bred, but of those cases in which one did, social status was key. In both experiments, however, we found no evidence that T concentrations co-varied with dominance or residence status in female juncos, that T concentrations were related to male preference for a female, or that T concentrations were related to the ability of females to initiate a nest. When we attempt to apply our findings to the field, we suggest that by aggressively defending residency within a territory, and possibly the male on the territory, resident/dominant females should be better able to secure nest sites, guard against nest parasitism, and keep exclusive paternal care for their young. If subordinate females were to remain on territories with the dominant female, they might be prevented from breeding. We conclude that aggression between females is likely to contribute to variation in female reproductive success in juncos and the maintenance of monogamy (as in the European starling, *Sturnus vulgaris*, Sandell, 1998; Sandell and Smith, 1997).

Our findings of a lack of correspondence between T, aggression, and dominance are similar to results from some species in which changes in female T have been investigated following simulated territory intrusions (Elekovich and Wingfield, 2000; Hau et al., 2004). But, several studies

comparing endogenous T in females with interaction frequency and population density have shown that females in denser groups that interact more often may have higher T (Hegner and Wingfield, 1987; Langmore et al., 2002; Mazuc et al., 2003; Smith et al., 2005; Whittingham and Schwabl, 2002, but see Schwabl et al., 1988). Furthermore, studies of female juncos have found that experimental increases in T can induce greater rates of aggression in females (Zysling et al., in review). While it is unclear exactly why differences in the findings concerning T and dominance or aggression in females exist, one possibility is that elevations in T occur only after multiple intrusions by several conspecifics, i.e., perhaps the need to repeatedly repel multiple intruders elevates T in females. Another possibility for the differences observed among studies could stem from the type of nesting resources being defended. Several of the species in which increases in T were associated with social interactions are colonial or semi-colonial cavity nesters (Hegner and Wingfield, 1987; Mazuc et al., 2003; Smith et al., 2005; Whittingham and Schwabl, 2002), while another has a polygynandrous mating system (Langmore et al., 2002). Both of these social systems may offer more chances for interactions over more limited reproductive resources than in a socially monogamous, territorial species and species of this type are predicted to have higher levels of circulating T in general (Møller et al., in press). Finally, results of implant studies with juncos may vary from the current findings due to the chronic elevation of T provided by implants. This suggests that T can influence aggressive behaviors in female juncos, perhaps directly or indirectly through its effects on other hormones, but naturally occurring levels of T may not normally respond to short-term stimulation by a conspecific competitor.

Another possible reason that T did not increase could be that female aggression is related to some other circulating hormone. Studies with other species have suggested that DHEA is important to non-breeding season aggression in males (Soma and Wingfield, 2001; Soma et al., 2002). Potentially, female aggression is mediated by DHEA and its conversion to other steroid hormones. Preliminary studies with female juncos did not find an increase in DHEA following aggressive interactions involving an intruder introduced to incubating females (Heidinger et al., unpublished data), but this possibility has not been explored at other stages of reproduction. Corticosterone (B) also may influence behavior during aggressive interactions (Nephew and Romero, 2003; Van Duyse et al., 2004), but previous work with juncos did not find significant increases in B during female resident–intruder introductions during the breeding season (Jawor et al., unpublished data). Progesterone (P), which could occur at high concentrations in females who are ovulating, may also influence aggression. P has been found to be influential in the developmental organization of aggressive behavior (Jennings et al., 2004) and possibly the initiation of adult aggression (Weiss and Moore, 2004) in male tree lizards (*Urosaurus ornatus*), but its effects on female intrasexual aggression are poorly studied. Hau et al. (2004) found that estradiol tended to increase with prolonged territory intrusions in spotted antbirds (*Hylophylax naevioides*), but not significantly. Yet, Elekonich and Wingfield (2000) found no

difference in estradiol (or T) between female song sparrows experiencing a simulated territory intrusion and birds caught passively. Juncos and song sparrows are in the same family (Emberizidae) and lack of a strong hormonal response in females to intrasexual aggression may be common in this family. Future studies will determine whether other steroids influence female aggression and dominance, and ultimately reproductive success in juncos.

Assuming that we are correct that female aggression related to pair formation is not directly influenced by variation in circulating T, we may ask why the situation differs from that in males. Female egg production is tightly linked to regular and precise hormonal fluctuations over a set time period (Johnson, 1986). Alterations in female hormonal titers (particularly luteinizing hormone) can alter timing of ovulation, egg size, and egg shell thickness (Gilbert et al., 1981). Additionally, elevated concentrations of circulating T in females may be reflected in elevated concentrations of T in egg yolks (Gil, 2003; Schwabl, 1996, 1997; Whittingham and Schwabl, 2002). Elevated concentrations of yolk T have been shown to have both beneficial and detrimental effects on offspring produced from eggs with high yolk T (reviewed in Gil, 2003; Groothuis and von Engelhardt, 2005). Given the potentially delicate relationships among circulating hormones, female fecundity, and yolk steroids, selection may have favored other mechanisms besides T to mediate female aggression at the time of pair formation.

We have presented here findings that intrasexual dominance status is important to female reproductive success in a socially monogamous species. Our findings suggest independence between female aggression and dominance and fluctuations in T, in contrast to the situation in males of many species. Recent interest has focused on the possibility that females may be a constraining force on males for the evolution of traits that benefit males but not females. In the case of T, males often benefit from higher levels of this hormone, while females may not (Ketterson et al., 2005; Møller et al., in press). Potentially, males and females have different activational mechanisms for a similar set of behaviors, a solution that circumvents potential detriments to females while allowing males to coordinate behavioral responses to other traits that benefit males.

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