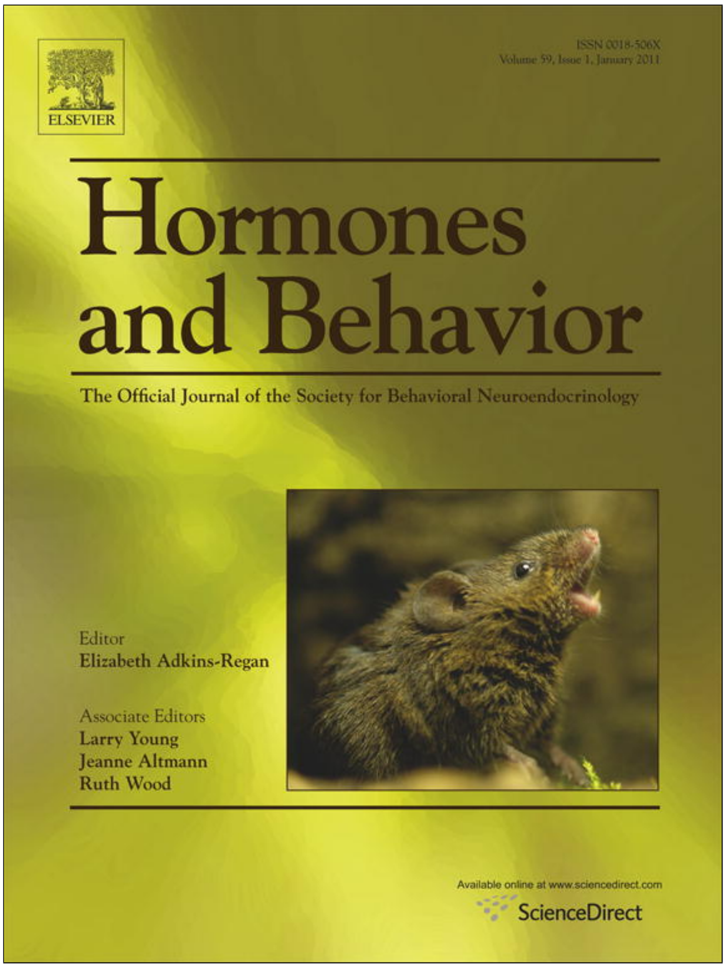
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Social environment affects testosterone level in captive male blue–black grassquits

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ABSTRACT

The challenge hypothesis proposes that testosterone (T) elevation above what is needed for breeding is associated with social factors, and males possibly modulate their hormonal response to variations in population density and sex ratio. We investigated the role of social environment in altering testosterone levels and aggression in a tropical, seasonally breeding grassquit (*Volatinia jacarina*). We exposed males to three social conditions during 1 year: all-males treatment (six males), mixed treatment (three males–three females), and paired treatment (one male–one female). We quantified aggressiveness among males and T plasma concentration for each individual in each treatment monthly. We found that more aggressive interactions occurred in the all-males treatment than in the mixed treatment. The data also revealed that, coincident with these behavioral changes, the patterns of T variation through time in each treatment were markedly different. The all-males treatment exhibited an early increase in T concentration, which was sustained for a lengthy period with two distinctive peaks, and subsequently declined sharply. The mixed treatment presented an intermediate pattern, with more gradual increase and decrease in T levels. At the other extreme, the paired treatment presented a later rise in T concentration. We conclude that the more competitive environment, with higher density of males, caused the early and higher elevation in T level, thus the presence of competitors may influence the decision of how much a male should invest in reproduction. We suggest that the male's perception of his social environment ultimately mediates hormonal production and alters his reproductive strategy.

Introduction

Many life-history traits can be better understood as resulting from tradeoffs associated with the physiological response of an organism to the challenges presented in its habitat (Wikelski and Ricklefs, 2001). Environmental, physiological, and social factors are important in triggering the beginning of reproduction. The steroid hormone testosterone (T) is a universal hormonal signal among male vertebrates to start investment in reproduction, resulting in morphological, physiological, and behavioral changes (Adkins-Regan, 2005). Plasma titers of T in male birds are almost undetectable during the non-breeding season but increase to breeding level in synchrony with gonad maturation, spermatogenesis, and development of secondary sex characteristics, in association with increasing frequency of typical sexual behavior (Wingfield et al., 1990). However, at different times and within distinctive contexts, males of many species may present higher levels of T than what is strictly necessary for reproduction.

The challenge hypothesis proposes that T elevation above the reproductive level is exclusively associated with social factors (Wingfield et al., 1990). Hence, T level variation may be an adaptive response to intra-sexual competition and/or inter-sexual interactions (Wingfield et al., 1990). In such social contexts, males possibly modulate their hormonal response to variations in demographic factors. Several laboratory studies have demonstrated that female presence (Moore, 1983; Pinxten et al., 2003), auditory and visual stimuli provided by other males (Small et al., 2008; Wingfield and Wada, 1989), and the visual stimulus of others males fighting (Oliveira et al., 2001) can increase T levels in males. However, few studies, either in the field or in laboratory conditions, have examined whether demographic factors such as population density or sex ratio play a role in regulating T concentration. Relative to sex ratio effects, for example, it is known that in many migratory bird species males arrive at the reproductive site before females, causing a transitory unbalanced sex ratio (Ketterson and Nolan, 1976). This occurs in the dark-eyed junco (*Junco hyemalis*) and results in an increase in male agonistic interactions; however, no changes in male T levels were found in the captive population studied (Greives et al., 2007). As for the effects of populational density, data are even more limited. In starlings (*Sturnus vulgaris*) and cliff swallows (*Petrochelidon pyrrhonota*), males in denser colonies presented higher T concentration than those in less crowded conditions (Ball and Wingfield, 1987; Smith et al., 2005). Thus, it appears that crowded conditions may lead to social instability associated with an increased number of territorial aggressions, and this may elevate T levels.

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The study of the reproductive physiology and behavior of tropical birds is still in its infancy, but data obtained to data present strikingly different patterns from those found for temperate birds (Martin and Rubenstein, 2008; Wikelski et al., 2003a,b). In the few tropical birds studied to date, those that exhibit year-round territoriality and long-term pair bonding do not exhibit high T levels, although territorial aggression is sustained (reviewed in Gwinner and Dittami, 1990; Goymann et al., 2004; Hau et al., 2000; Wikelski et al., 2000). The key factor underlying life-history traits of tropical birds is the duration of the breeding season, which may be the chief determinant of their low T levels: if the breeding season is short, the T peak should be higher, but if the breeding season is long, the T peak is expected to be lower (Goymann et al., 2004). This happens because the maintenance of high and prolonged T levels may have an excessive physiological cost to the individual (Wingfield et al., 2001). Additionally, recent data are also pointing toward alternative hormonal mechanisms that could regulate the territorial aggression typically seen in tropical birds, such as the role of DHEA (an androgen precursor), which would supplant T without the costs of the latter (Hau et al., 2004; Wingfield et al., 2001).

In this study, we were interested in examining the impacts of prolonged exposure to specific social environments on male behavior and physiology. To this end, we investigated T levels and aggression in response to social environment in captive blue–black grassquits (*Volatinia jacarina*). This neotropical passerine provided an excellent model for examining this issue due to the dynamics of its annual cycle in the wild, wherein individuals are exposed to changing social contexts. Male blue–black grassquits spend a large part of their pre-breeding phase in mixed-species flocks of males and females. They subsequently interact aggressively with other males to demarcate territories, and this is then followed by pairing with females for reproduction. We experimentally tested whether social environment is causally responsible for T levels by exposing captive males over a long-term period to different treatments that varied in density and sex ratio. Three treatments were used: groups of only males, mixed groups with equivalent number of males and females, and isolated paired males with females. We hypothesized that higher levels of aggressiveness would occur in treatments where males interacted with other males, when compared with the treatment where males were paired with females. We expected that males in treatments with higher rates of aggressive interactions would also exhibit higher circulating levels of T. Taking into consideration the two treatments where males could interact with other males, we thought it more likely that the mixed group would exhibit more aggressive encounters among males, since they might be competing for females, and thus would also have higher T levels.

Materials and methods

Study animals and housing

Blue–black grassquits are small, granivorous birds (family Emberizidae) with an extensive geographic distribution from Mexico to Chile (Sick, 1997). In central Brazil, where this study was conducted, these birds are migratory, arriving in the area each year starting in October, breeding from about November to April, after which they depart (Carvalho et al., 2007). Adult males exhibit plumage that varies from glossy black, during the breeding season, to black with brown patches, outside the breeding season (Aguilar et al., 2008), while females and juveniles have brown, inconspicuous plumage. The males defend small territories during the breeding season, presenting a conspicuous, multi-modal display of repeated jumps from elevated perches, which are powered by wing-flapping, while exhibiting a white under-wing patch and vocalizing. Nests are built by each pair inside the male's territory and both male and female present parental care (Carvalho et al., 2007). The blue–black grassquit is socially monogamous (Almeida and Macedo, 2001), and recent studies have found a high rate of extra-pair fertilization (60% of chicks) for our study population (Carvalho et al., 2006).

Between February and March 2007, we captured 46 male and 22 female blue–black grassquits with mists nets in the Universidade de Brasília campus (15°46'S, 47°52' W), central Brazil. The climate of the region is seasonal, with a rainy period from October to April and a dry season from May to September. In free-living grassquits, breeding typically coincides with the rainy season. The birds were kept in an outside aviary, and males and females were placed in individual cages that were visually isolated from each other until June (see below). We banded each male with numbered metal bands provided by the Brazilian bird-banding institution, Centro Nacional de Pesquisa para Conservação das Aves–CEMAVE. All birds were provided with an *ad libitum* diet of a mixture of seeds (green, red, and yellow millet and birdseed), in addition to sterilized sand and water fortified with a vitamin complex. All individuals were treated with vermifuge (mebendazole) and medication to control for ectoparasites (sulphametoxazol–trimethoprim, pirethrin talc). Capture, aviary maintenance, and banding procedures were authorized by CEMAVE (license no. 1301/2) and Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renováveis (IBAMA license no. 206/2006).

Experimental design

The experimental design involved separating the birds into the following treatments: (1) three replicates of six males ("all-males treatment"), each replicate housed in a separate compartment (2.6 × 1.5 × 2.0 m); (2) three replicates of three males and three females ("mixed treatment"), each housed in a separate compartment (2.6 × 1.5 × 2.0 m); and nine replicates of one male and one female ("paired treatment"), each housed in a separate cage (55 × 30 × 38 cm). Replicates of all treatments were visually although not acoustically isolated. Each treatment had a backup group, kept in similar housing and feeding conditions and equivalent size replicates, to be used in case we had to eliminate a replicate.

We initiated the experiment with 36 males in the three treatment groups, but during the 1-year experimental period (July 2007–July 2008), 10 males died between July and September 2008 of unknown causes, although the aviary population typically presents high mortality during the dry season. Because of the differential mortality for some replicates, we eventually had two of the three all male replicates with four males instead of the original six, and in one of the mixed replicates, we had two males instead of the original three. Thus, final numbers included a total of 14 males in the all-males treatment (subdivided in three replicates) and eight males in the mixed treatment (subdivided in three replicates). Data on aggression were standardized by dividing measures by the number of individuals in each trial.

Hormone assays

From July 2007 to July 2008, we took small blood samples (approximately 150 μl) monthly from males by puncturing the alar vein, preferentially during the first week of the month and early in the morning (0600 h), immediately after which they were centrifuged at 11,500 rpm. The plasma was then stored at −20 °C until assay. Sufficient plasma for T assays was obtained from 40 males, totaling 388 blood samples during 12 months. The T concentration was measured by the method of radioimmunoassay, using an antibody for T (reference #RDI-TRK2T2) from Research Diagnostics Inc. (Flanders, USA) and following the procedure described by Wingfield et al. (1992). The cross-reactivities provided by the supplier are 16% for 5-α-dehydrotestosterone, 1% for androstendiol, 0.4% for androstendione, <0.1% for androsterone, <0.1% dehydroepiandrosterone, <0.1% for progesterone, <0.01% for estradiol/estriol, and 0.01% for cortisol/pregnenolone. The detection limits of the assays were between 0.20 and 5.00 ng/ml. The samples were divided in seven assays. The intraassay coefficient of variation (CV) was 2.3%, and the inter-assay coefficient of variation was 9.9%.

Aggressive social interactions

We determined levels of aggression monthly for each replicate in the two treatments that included multiple males (all males and mixed) during 9 months (November 2007–July 2008). For each replicate, we evaluated the rate of aggressive encounters among males over a food source during experimental trials. The night before the trial, the males were taken to an indoor laboratory, where they were deprived of food (but not water). At 0600 h on the following morning, the males from each replicate were placed in a neutral interaction arena (60 × 45 × 60 cm) inside which a single food source was supplied in a central position. Trials lasted for 15 min and were recorded with a video camera. Typically, a male approached the feeder and displaced another through aggressive interactions, which included stereotyped behavior wherein both males faced each other with lowered head and open beak, and in some occasions, this was followed by pecking and chasing. The monthly agonistic interaction value for a replicate was obtained by adding all aggressive interactions observed during the trial and dividing by the number of males in the replicate.

Statistical analyses

Since hormonal levels may vary temporally in complex, nonlinear fashion, we used a generalized additive mixed model (GAMM) approach. GAMMs allow for the fitting of models that include parametric and smooth terms as covariates (Wood, 2006), as well as random effects to account for data non-independency. Testosterone concentration (ng/ml) was log-transformed and included as the response variable; social treatment group was included as a fixed term, and the time effect was modeled as a penalized regression spline, with smoothed parameters selected by maximum likelihood (Wood, 2006). Individual bird identity was included as a random effect (but see below). A similar model framework was used to test for the effects of social context and time on social interactions, but since in this case social interactions were averaged by replicate, only replicate cage identity was included as a random factor. To test if treatment effects in aggression rates were a result of long-term effects of the social environment or short-term density effects, we modeled the number of aggressive interactions only in those groups that fluctuated in size (due to deaths; see above) as a function of time and number of individuals, with cage identity as a random factor. In this analysis, the effects of both time and number of individuals were modeled as splines, to allow for nonlinear effects of time and density in mean aggression.

The interaction between social group (treatment) and the smoothed term for time was modeled using variable coefficient models (Hastie and Tibshirani, 1993). Basically, these models allow for smooth terms conditioned to specific values of the parametric covariate—that is, in our case, for the modeling of treatment-specific responses through time (Wood, 2006). Parsimonious models were obtained through a backward stepwise procedure, by sequentially removing covariates (starting with higher order interactions). Differences in model fit were estimated through likelihood ratio tests (LRT), using the difference in deviance as a Chi-squared approximation (Wood, 2006) and keeping the simpler model whenever the removal of the variable caused no significant decrease in model fit (Quinn and Keough, 2002).

In addition to the repeated measures of individual birds, we considered and took into account another source of non-independent data as a random term: birds housed in the same cages for the "all-males" and "mixed" treatment levels may have been subjected to similar conditions that affected the variables measured. However, in order to avoid overfitting our models by estimating parameters without improving model adherence, we tested the significance of this random effect both on initial and final models. Since in all cases adding this nesting specification had negligible effect (LRT, all $P > 0.9$), data were considered independent for group cage identity, and we only included the individual identity random effect in our models with testosterone concentration as the response variable.

We excluded 10 males that died in the first 3 months, and for this reason, the time frame considered in our models included only the months from November 2007 to July 2008. A total of 31 males were included in the model through a period of 9 months.

Results

Males in the two treatments with multiple males exhibited differences in the absolute number of aggressive interactions ($\chi^2 = 6.83$, $df = 1$, $P = 0.009$). The highest number of aggressive interactions (during 15-min trials) was found for the all-males treatment (monthly mean = 9.4 ± 1.2, $n = 9$ months), while the mixed treatment had about one-third this number of aggressive encounters (monthly mean = 3.8 ± 1.1, $n = 9$ months; Fig. 1). There was no significant variation in the number of aggressive interactions through time for either of the treatments ($\chi^2 = 4.14$, $df = 2$, $P = 0.13$). Considering only the groups that fluctuated in size, the number of aggressive interactions was not associated to group size (estimated degrees of freedom for the number of individuals smooth parameter = 1, indicating a linear relation; $\chi^2 = 0.72$, $df = 2$, $P = 0.69$).

Considering the entire 12 months of the experiment (July 2007 to June 2008), the mean T concentration in male blood plasma was 0.60 ± 0.01 ng/ml ($n = 40$ males: includes 36 males that initiated experiment plus four early substitutes). The mean T concentration in the non-breeding season (mid-May to September) was 0.54 ± 0.02 ng/ml ($n = 40$ males) and during the breeding season (December–April) was 0.62 ± 0.01 ng/ml ($n = 37$ males). The highest T concentration level found for a single male occurred in an individual in the all-males treatment and was 2.33 ng/ml while the lowest concentration was 0.20 ng/ml from a male in the mixed treatment. Two samples were below the assays detection limits of 0.20 ng/ml.

We found significant variation of T levels through time ($\chi^2 = 120.42$, $df = 2$, $P < 0.0001$), with all treatments presenting a large and relatively sharp increase in T level during the breeding period, followed by a decline when leaving the reproductive condition (around April in central Brazil; Fig. 2). Although there was no significant and absolute difference between treatments ($\chi^2 = 2.7$, $df = 2$, $P = 0.26$), T levels varied differently through time in the treatments ($\chi^2 = 14.21$, $df = 4$, $P = 0.007$). T levels in the all-males treatment rose early and abruptly (first peak in February) when compared with the other treatments and remained at a high level for 4 months before starting to decline (May). The T peak for this treatment was considerably higher (0.87 ng/ml) than that found for the other two treatments (mixed: 0.75 ng/ml; paired: 0.75 ng/ml). Additionally, the all-

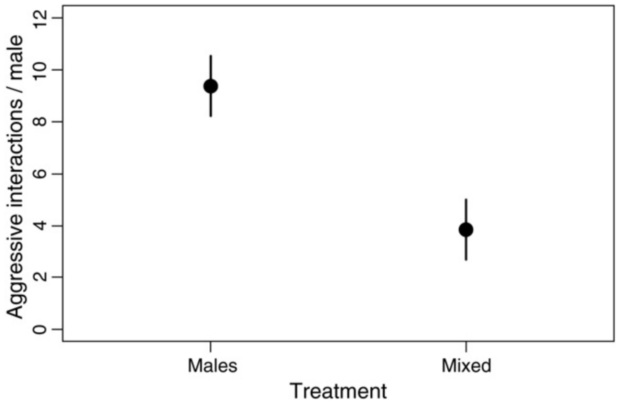

Fig. 1. Treatment differences in frequency of aggressive interactions among blue–black grassquit males during 15-min trials (mean ± 1 SE) for males in the all-males and the mixed treatment groups. The all-males treatment had significantly more aggressive interactions than the mixed treatment ($P = 0.009$).

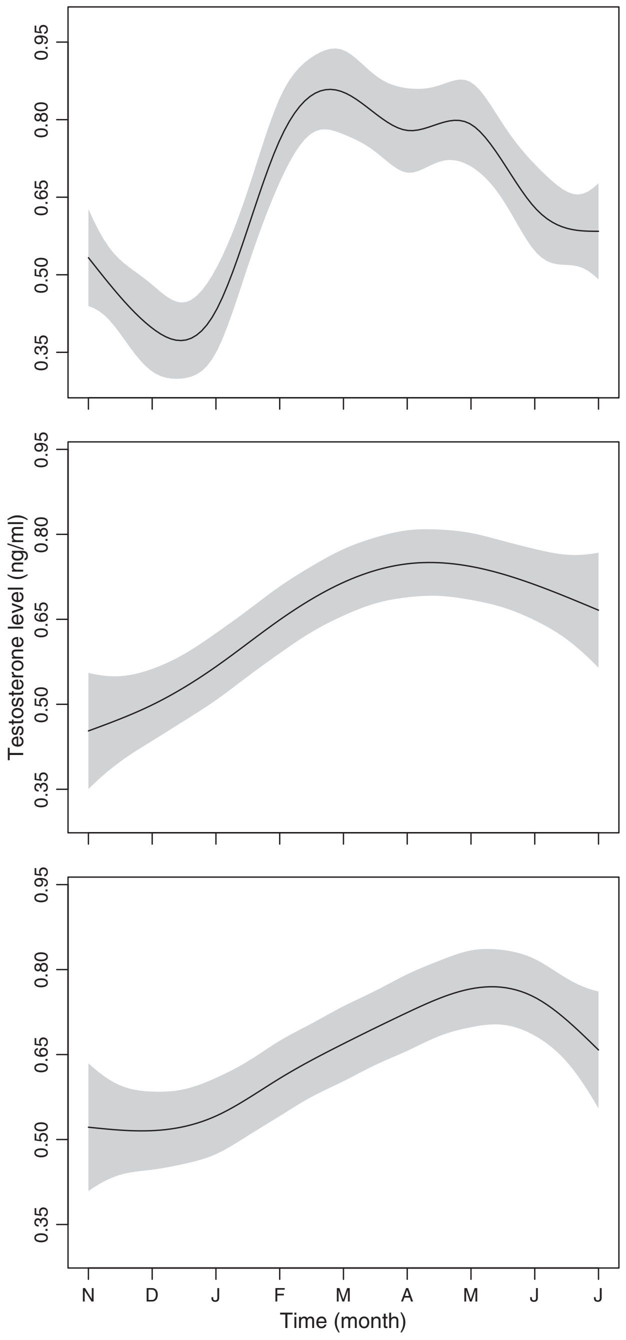

Fig. 2. Level of T concentration from November 2007 to July 2008 in male blue–black grassquits maintained in three treatments: all males, mixed, and paired. Testosterone peaked in all three treatments during the breeding season and showed significant variation through time between treatments. T concentration shown in absolute values (ng/ml), which were log-transformed for statistical analyses. The effect of time on T concentration was estimated as a smoothed term using penalized regression splines in a General Additive Model with variable coefficients (see Materials and methods for details); the grey shading represents the 95% confidence interval.

males treatment exhibited two peaks in T wherein even the lowest value between peaks was still as high as the peak values for the other two treatments. In the mixed treatment, T levels climbed more gradually and took considerably longer to peak (April) and then declined more slowly. Finally, in the paired treatment, T level only reached a peak in late May, much later than the others treatments (Fig. 2). As withdrawal of the two samples below the assays limits of detection did not significantly alter the results (time, $\chi^2 = 144.34$, $df = 2$, $P < 0.001$; treatments, $\chi^2 = 3.14$, $df = 2$, $P = 0.21$; interaction, $\chi^2 = 11.20$, $df = 4$, $P < 0.02$), these were retained in the analysis.

Discussion

The three experimental treatments in this study (all males, mixed, and paired) presented significant variations in T concentration through time, and as could be expected, males generally presented a high level of T during the breeding season, followed by a decline that coincided with the end of the rainy period in central Brazil. This is a clear indication that the experimental conditions of captivity did not interfere with the typical physiological response to external conditions that precipitate breeding readiness. But beyond the typical and cyclical T variations associated with seasonality, in this experiment, we demonstrated that T levels may also be dependent upon social context. The all-males treatment exhibited an early T level increase when compared to the other treatments and also had two distinctive peaks, characterizing a plateau where T remained at high concentrations. Although breeding condition of males in the all-males treatment ended at about the same time as for males in the other treatments, the decline in T was much sharper. The mixed treatment presented a pattern, with more gradual increase and decrease in T levels, and no elevated plateau. At the other extreme, the paired treatment males presented a later peak in T levels, only noticeable in April. Thus, we here demonstrate that there is a causal association between social environment of males and the pattern of T concentration within the general physiological framework that reflects seasonal breeding condition.

In this study, we observed that individuals in the all-males treatment were involved in a higher number of aggressions when compared to those in the mixed treatment, and that this difference was not influenced by reproductive condition (seasonality). This was surprising since in this treatment, males were not competing for access to females nor for food throughout the experimental long-term period. It is known that social challenges between males can lead to an increase in T level (Goymann et al., 2007; Wingfield et al., 1990), thus we expected that the treatment that presented higher aggression levels would also have higher T levels. Our experiment sustains this expectation, but in a rather unusual way. Unexpectedly, the elevated number of aggressive interactions in the all-males treatment may also have precipitated an advanced readiness for breeding, i.e., a higher T elevation much earlier in time, relative to other treatments. The sex ratio in the mixed treatment may provide an explanation for the lower aggression we found: the equal number of males and females allowed each male access to at least one female, leading to lower aggression compared to the all-males treatment, where there was no access to females. Additionally, during the competitive trials over food, the replicates from the all-males treatment typically involved a higher number of males when compared to the mixed treatment replicates. Although the averages for the analyses were obtained by dividing aggressive interactions by the total number of males in each trial, the simple presence of more males may have induced higher aggression levels. On the other hand, the males from the paired treatment, devoid of aggressive interactions altogether, took longer to attain a peak in T concentration and generally maintained lower levels of T through time.

Testosterone is usually associated with a male's total investment in reproduction, because in most cases, this hormone mediates the acquisition and maintenance of secondary sex characteristics (McGlothlin et al., 2008; Peters et al., 2000), in addition to muscle hypertrophy, spermatogenesis, territoriality, singing, and displaying (Wingfield et al., 2001). Previous studies have found that testosterone production quite often responds to environmental conditions (photoperiod), in preparation for breeding (reviewed in Dawson, 2008) and, possibly, social factors as well. For example, in a study of cliff swallows (*P. pyrrhonota*), density was associated with T concentrations, and males in larger colonies presented higher T levels when compared to those in smaller colonies, and this difference occurred only during the breeding season (Smith et al., 2005). However, the level of aggressive interactions between males was not measured, but the authors attribute the results to the fact that, in larger colonies, there was more intra-sexual competition. In our study, we could not verify whether there is a positive association between individual levels of T and aggression, because we measured number of aggressive interactions and disregarded who initiated the interaction itself. It is important to highlight that characteristics of a single individual may influence a group's outcome. For example, hyperaggressive males within complex networks of interactions have been identified in some species (Sih and Bell, 2008), and such individuals could possibly determine an overall higher number of aggressions within a given group.

The manipulation of sex ratio in captive groups of dark-eyed juncos (*J. hyemalis*) was not associated with differences in male T level, despite the fact that the male-biased treatment had a higher number of social challenges (Greives et al., 2007). Results relative to T in this case may reflect the fact that sampling occurred at only a single point in time, therefore excluding the time factor from analysis, which may have obscured possible differences in the patterns of T production over time. In the present study, we observed that the more competitive environments, with higher density of males, resulted in an early elevation in T level and a longer duration in sustaining high values of this hormone. This indicates that the occurrence and density of competitors trigger male investment in reproduction and may be more important in the male physiological decision-making process than simple female presence.

Maintenance of high T levels may be costly to males, because this hormone suppresses the immune system, increases metabolic rate and energetic expenditure, reduces fat reserves, and finally, stimulates aggressiveness and willingness to engage in physical disputes, increasing the probability of injury or death (Folstad and Karter, 1992; Wingfield et al., 2001). The abrupt decrease in T levels shown in the all-males treatment group at the end of the breeding period may be a strategy to avoid the deleterious costs of maintaining high levels of T. Males in this treatment sustained high T levels for 4 months, considerably longer than males in the other treatments. In the others two treatments where males upheld high T for less time, the decline in hormone concentration was slower and smoother.

Conclusions

Our objective in this study was to determine how external factors, more specifically, social context and interactions over a prolonged period of time, affect patterns of aggressiveness and T concentrations, both of which can mediate male reproductive strategy. Overall, we found that social environment influences the patterns of male T secretion over time and that social instability, due to higher levels of competitive interactions, is the probable causal element. Competitive interactions, contrary to expectations, were significantly inflated in the environment where males were not allowed access to females. Moreover, we found that males in the all-males treatment attained a physiological breeding state even in the absence of females.

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