Natural Variation in a Testosterone-Mediated Trade-Off between Mating Effort and Parental Effort

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abstract: Male birds frequently face a trade-off between acquiring mates and caring for offspring. Hormone manipulation studies indicate that testosterone often mediates this trade-off, increasing mating effort while decreasing parental effort. Little is known, however, about individual covariation between testosterone and relevant behavior on which selection might act. Using wild, male dark-eyed juncos (*Junco hyemalis*), we measured individual variation in testosterone levels before and after standardized injections of gonadotropin-releasing hormone (GnRH). The GnRH challenges have been shown to produce short-term testosterone increases that are similar to those produced naturally in response to social stimuli, repeatable in magnitude, and greater in males with more attractive ornaments. We correlated these testosterone increases with behavioral measures of mating and parental effort (aggressive response to a simulated territorial intrusion and nestling feeding, respectively). Males that showed higher postchallenge testosterone displayed more territorial behavior, and males that produced higher testosterone increases above initial levels displayed reduced parental behavior. Initial testosterone levels were positively but nonsignificantly correlated with aggression but did not predict parental behavior. These relationships suggest that natural variation in testosterone, specifically the production of short-term increases, may underlie individual variation in the mating effort/parental effort trade-off. We discuss the implications of these results for the evolution of hormonally mediated trade-offs.

Keywords: testosterone, life-history trade-offs, sexual selection, territorial aggression, parental care, *Junco hyemalis*.

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Life-history trade-offs arise when traits that contribute to fitness are inversely linked and are thus inhibited from evolving independently (Stearns 1992; Roff 2002). When a trade-off is present, multiple beneficial traits cannot be maximized simultaneously, at least in the short term, and theory predicts that selection will favor the optimal combination of traits within the constraints imposed by the trade-off (Stearns 1992; Roff 2002; Roff and Fairbairn 2007). One of the most common trade-offs in animals involves mating effort (the amount of energy, time, or other key resources invested in competing for mates) and parental effort (the amount of resources invested in rearing offspring; Magrath and Komdeur 2003). In many species, such as biparental birds with extrapair fertilizations, this trade-off is particularly important because mating effort and parental care may overlap in time. Hence, investment in one activity usually requires reduced investment in the other.

Trade-offs are often mediated by physiological factors such as hormones, and understanding their physiological basis may provide insight into life-history evolution (Stearns 1992; Finch and Rose 1995; Sinervo and Svensson 1998; Ketterson and Nolan 1999; Zera and Harshman 2001; Ricklefs and Wikelski 2002; Adkins-Regan 2005; Hau 2007). Hormonal manipulations provide a powerful means to dissect the mechanistic basis of life-history trade-offs and to experimentally test for effects on fitness, especially when they are used in long-term studies of natural populations (Ketterson et al. 1996; Reed et al. 2006). However, to understand the evolution of hormone-related trade-offs more fully, we must also consider naturally occurring variation among individuals, which must be present in order for selection to act (Adkins-Regan 2005). Such variation may lie in the strength of the hormonal signal, the sensitivity of the individual to the hormones, or both. Empirically, a logical first step is to focus on hormone concentrations in the circulation, which are often much easier to measure than individual sensitivity.

Examining individual variation in the physiological ba-

sis of trade-offs involving male mating effort is of considerable interest because theory predicts that males should invest differentially in relation to their quality (Nur and Hasson 1984; Getty 1998, 2006). The potential benefit of increasing mating effort at the expense of parental effort is often great, as total reproductive success tends to increase with mating success in males (Trivers 1972; Arnold 1994; Queller 1997; Wade and Shuster 2002; Shuster and Wade 2003). However, males must compete among themselves for a limited number of mating opportunities, and one male's mating success necessarily comes at the expense of another male. As a consequence, the variance in male reproductive success increases as the maximum number of mates increases (Shuster and Wade 2003). Because all males cannot succeed equally at obtaining multiple mates, males may differ in their optimal investment in mating effort (Trivers 1972; Nur and Hasson 1984; Getty 1998, 2006). Males that are more likely to succeed in obtaining mates because, for example, they possess a more attractive ornament, would benefit from increased investment in mating, whereas males that are less likely to be successful at mating may benefit more from investing in parental care. Selection may thus maintain variation in the resolution of this trade-off as well as its covariation with male attractiveness or quality (Getty 2006; Roff and Fairbairn 2007).

In birds, the trade-off between mating effort and parental effort appears to be mediated, at least in part, by the steroid hormone testosterone (Ketterson and Nolan 1992, 1994, 1999; Adkins-Regan 2005; Hau 2007). Many studies that have used experimental elevation of testosterone by means of subcutaneous implants have shown increases in mating behavior and decreases in parental behavior (e.g., Silverin 1980; Wingfield 1984; Hegner and Wingfield 1987; Dittami et al. 1991; Ketterson et al. 1992; Raouf et al. 1997; Van Roo 2004; but see Hunt et al. 1999; Van Duyse et al. 2000, 2002; Lynn et al. 2002, 2005). However, it has been difficult to study individual-level variation in testosterone and the behaviors it mediates, perhaps because testosterone varies so much within individuals (Adkins-Regan 2005). In many songbirds, testosterone levels show temporal variation on both relatively long-term (seasonal) and short-term scales (Wingfield et al. 1990). Short-term changes are particularly interesting because in many species, they are induced by social stimuli and occur during the production of mate-acquisition behavior, such as territorial aggression and courtship (Harding 1981; Moore 1983; Wingfield 1985; Wingfield et al. 1990, 2001; Pinxten et al. 2003; but see Van Duyse et al. 2004; Landys et al. 2007; Lynn et al. 2007). Because of this association with behavior, variation in transient testosterone elevations may be more relevant to the mating effort/parental effort

trade-off than is baseline circulating testosterone. To our knowledge, no studies have examined this relationship.

We assessed natural covariation between testosterone and behavior in a songbird, the dark-eyed junco (*Junco hyemalis*). A long-term implantation study has shown that testosterone generally increases mating effort and mating success while decreasing parental behavior (Ketterson et al. 1992; Enstrom et al. 1997; Raouf et al. 1997; Cawthorn et al. 1998; Schoech et al. 1998; Reed et al. 2006). Natural testosterone levels show long-term and short-term variation, with males transiently increasing testosterone levels during territorial interactions (Ketterson and Nolan 1992; Jawor et al. 2006; J. W. McGlothlin, J. M. Jawor, T. J. Greives, J. M. Casto, J. L. Phillips, and E. D. Ketterson, unpublished manuscript). The magnitude of short-term testosterone increases can be measured using gonadotropin-releasing hormone (GnRH) challenges, which are simple bioassays that are often used to measure the responsiveness of the hypothalamo-pituitary-gonadal (HPG) axis, which regulates testosterone production (e.g. Millesi et al. 2002). Testosterone levels produced by male juncos in response to GnRH challenges are correlated with those produced during territorial interactions, and the magnitude of the testosterone increase varies among individuals (Jawor et al. 2006; J. W. McGlothlin, J. M. Jawor, T. J. Greives, J. M. Casto, J. L. Phillips, and E. D. Ketterson, unpublished manuscript). Further, males with larger plumage ornaments (a white patch on the tail) produce larger testosterone increases when injected with GnRH (J. W. McGlothlin, J. M. Jawor, T. J. Greives, J. M. Casto, J. L. Phillips, and E. D. Ketterson, unpublished manuscript).

Following from these results, we predicted that variation in the responsiveness of the HPG axis, and thus the ability to produce short-term testosterone increases, might account for natural variation in relative allocation to mating effort versus parental effort. To test this prediction, we measured natural covariation between behavior (as a proxy for effort) and the response to GnRH challenges. As a measure of mating effort, we assessed aggression during simulated territorial intrusions and correlated this behavior with GnRH challenges performed during early breeding. Territorial aggression is a major component of competition for mates in birds and is thus likely to contribute to variation in mating success. In two separate breeding seasons, we assessed nestling feeding rate as a measure of parental effort, correlating it with GnRH challenges performed within 1 day of the behavioral observations. Male juncos do not incubate, and thus nestling feeding represents their major contribution to parental care. If variation in the capacity of the HPG axis to generate acute increases of testosterone underlies variation in the trade-off between mating effort and parental effort, we expected to find that response to GnRH challenges should correlate positively

with aggressive behavior and negatively with parental behavior.

Methods

Study Species and General Methods

We studied a wild breeding population of Carolina dark-eyed juncos (*Junco hyemalis carolinensis*) near the University of Virginia's Mountain Lake Biological Station in Giles County, Virginia (37°22′N, 80°32′W). Nolan et al. (2002) provide a detailed account of junco breeding biology. Briefly, at the beginning of the breeding season (late March–early April), male juncos establish and defend all-purpose territories. Females build nests and begin to lay eggs in late April. Females incubate clutches of three to five eggs for an average of 12 days. Both sexes defend the nest from predators, feed nestlings after hatching for an average of 12 days, and feed fledglings after they leave the nest. Juncos in our population repeatedly renest if nests are lost and attempt additional nests following nest success (up to three successful nests in a single season). Extrapair fertilizations are common and may account for up to 56% of young (Nolan et al. 2002).

The population was censused at the beginning of each breeding season by capturing adults in baited mist nests and Potter traps, the locations of which remained the same each year. At each capture, standard morphometric measurements were obtained from each individual. If a bird had not been captured previously, it was marked with a U.S. Fish and Wildlife Service leg band and a unique set of plastic color bands so that it could be identified from a distance. Sex was determined using cloacal protuberance (males) or brood patch (females) development when possible; otherwise, larger birds were classified as males. Such an assignment was later confirmed by sexual development and/or behavior.

When birds began nesting, we attempted to find all nests in our study area, using systematic searches and by observing focal bird behavior. Once located, nests were visited every 1–3 days thereafter to monitor progress. We assigned each nest to a pair of adults based on their behavior at the nest.

Hormone Sampling

To assess natural variation in circulating testosterone and the sensitivity of the HPG axis, we used intramuscular injections of GnRH. Such GnRH challenges induce the pituitary to release luteinizing hormone into circulation, which in turn stimulates the testes to release testosterone. In male juncos, GnRH challenges induce peak testosterone levels at 30 min postchallenge, and the levels return to baseline after 2 h (Jawor et al. 2006). Importantly, testosterone levels produced after a GnRH challenge are correlated with natural increases in testosterone produced in response to a territorial intruder (J. W. McGlothlin, J. M. Jawor, T. J. Greives, J. M. Casto, J. L. Phillips, and E. D. Ketterson, unpublished manuscript).

After a bird was captured, it was returned to a central laboratory where a blood sample (∼100 μL) was taken from the wing vein to measure initial testosterone level. We measured handling time (min), which was controlled in the statistical models, as the time elapsed between capture and the collection of this blood sample. Afterward, 50 μL of a solution containing 1.25 μg of chicken GnRH-I (Sigma L0637; American Peptide 54-8-23) dissolved in 0.1 M phosphate-buffered saline solution was injected in the left pectoralis major. The bird was immediately placed into a holding bag. Exactly 30 min after the injection, a second (∼100 μL) blood sample was taken to measure post-GnRH-challenge testosterone. Blood samples were centrifuged, and the plasma fraction was reserved and frozen at −20°C until assayed. The sampling regime for these challenges differed among years according to the type of behavior being measured.

Territorial Aggression

In 2006, we performed GnRH challenges on males caught at random during the early breeding season (April 12–May 17). Because the intensity of GnRH-challenge response may decrease within males as the breeding season progresses (Jawor et al. 2006), we attempted to perform two GnRH challenges on each male found on our study site to obtain an average response for each male. For a given male, the second challenge was performed 6–29 days (mean 12.8) after the first. In total, we performed 173 GnRH challenges on 114 different males. For 36 males, we were able to locate territories and measure territorial aggression. Twenty-one of these males had previously received two GnRH challenges, separated by 7–22 days (mean 13.3), and 15 had received a single challenge. For a given male, behavioral measurements were collected 9–40 days (mean 23.7) after a male's first GnRH challenge and 3–26 days (mean 13.3) after its second challenge (if any). Handling time for these samples ranged from 8 to 96 min (mean 35).

We used observations of behavior, often stimulated by brief song playback (1–3 min), in order to map the territories of males. Although the period of territory mapping overlapped temporally with both hormone sampling and measurement of territorial behavior, these activities were never conducted in the same part of the study site on the same day.

Simulated territorial intrusions were conducted between

April 29 and May 29 to measure territorial aggression. In each intrusion, we placed a captive lure male in a small cage in the estimated center of the focal male's territory. The captive lures ($n = 8$) used in this study were captured from areas that were at least 3 km away from our study site, so they were unlikely to be familiar or related to focal males. The cage was covered with a cloth until the trial began. Two nylon ropes with plastic flagging placed at distances of 5 and 10 m, which were attached to the bottom of the cage, were stretched along the ground in opposite directions and used to judge distance of the focal male from the cage. A portable compact disc player (Duraband CD-855) attached to a battery-powered speaker (Radio Shack 40-1441) was placed directly next to the cage.

At the beginning of each trial, the speaker was set to full volume, the player was started, and the cover was removed from the captive male's cage. The two observers then retreated to a location at least 15 m away from the cage. After 5 min of silence, a 15-min recording of junco long-range song (Titus 1998) was played. The recording consisted of five different song types, recorded in our study population $\geq$10 years before. Each song type was repeated nine times at a rate of 6 songs min^{-1} (for a total of 45 songs in 7.5 min), and then this series was played a second time (90 songs in 15 min). Audacity 1.2.3 for Windows (http://audacity.sourceforge.net) was used to compile the composite recording, remove background noise, and equalize volume of different song types. The sound power level was 92 dB, measured at 1 m using a sound level meter (Radio Shack 33-2050). This sound level is comparable to a junco singing a long-range song (Nolan et al. 2002).

We recorded four variables related to territorial aggression. First, latency was the amount of time (in s, recorded using a stopwatch) between the beginning of the song playback and when the male was first seen or heard. Seven males that approached the lure before the song began were assigned zero latency. We used a second stopwatch to record the time (in s) spent within 5 m of the cage. We counted the number of flyovers (a flight directly over the lure's cage) and the number of long-range songs produced. In each trial, one observer (J. W. McGlothlin) watched the bird through binoculars and noted behavior, while a second operated the stopwatches, recorded the behavior on a data sheet, and helped locate the bird if needed.

If a male did not appear in response to the stimuli, the trial was not used. Trials were abandoned or discarded if the responding male could not be identified by its color bands. We did not perform simulated territorial intrusions on males that were known to be feeding nestlings, and we excluded data from four males known to be feeding fledglings because of the dramatic changes in both behavior and home range at that stage of reproduction (Nolan et al. 2002). Of the remaining trials conducted on 36 males, four of the males had mates that had not yet produced an egg, five had mates that were incubating (male juncos do not incubate), eight had recently lost nests to predators, and 19 were of unknown nesting stage. Territorial behavior (first principal component; see "Statistical Analyses") did not differ statistically among these groups ($P = .65$).

Parental Behavior

In 2003–2004, we measured parental behavior and GnRH-challenge response in 24 males that were feeding nestlings (12 in 2003, 13 in 2004; one male was measured in both years). Each brood had two to five nestlings (mean 3.5). Family size was reduced in five of the nests observed because we collected an egg for steroid analysis as part of another study.

We temporarily removed a male's mate when measuring the male's parental behavior in order to control for potential interactions between members of the pair (Clotfelter et al. 2007). These females were caught using a mist net in front of the nest. After catching the female, we counted and weighed the nestlings and placed a video camera on a tripod near the nest. Females were held in the laboratory in individual cages and provided with food and water ad lib. Male behavior at the nest was recorded for 4 h, at which time the female was returned to the site of capture and released. Recordings of parental behavior were made on either day 6 or day 7 after hatching, and all recordings were begun between 0600 and 1100 hours. Videotapes were scored for number of visits to the nest with food, and and that was divided by total recording time to calculate feeding rate.

To measure GnRH-challenge response in relation to parental behavior, we caught males using the same mist net arrangement used to capture females. Most males were caught the day after their behavior was recorded ($n = 20$). However, if we were unable to catch the female on day 6 and instead caught the male, the male was given a GnRH challenge on day 6 and assessed for parental behavior the following day ($n = 5$). We did not include day of capture in our statistical analyses; however, controlling for this capture factor did not affect our results.

Males were returned to the laboratory to receive GnRH challenges according to the procedure described above. Handling time ranged from 14 to 217 min (mean 60). Following the challenge, the male was temporarily housed in a small cage (approximately 4 h) and provided with food (white millet and mealworms) and water while female parental behavior was being measured as part of another study.

Testosterone Assays

Plasma collected from GnRH challenges was analyzed for testosterone measured using an enzyme immunoassay (EIA) kit (901-065, Assay Designs). Assay methods are described in detail elsewhere (Clotfelter et al. 2004). Approximately 2,000 counts per minute of tritiated testosterone were added to each sample in order to calculate recoveries after two extractions with diethyl ether. Extracts were resuspended in 50 μL ethanol and diluted to 350 μL with assay buffer from the kit. From each reconstituted sample, 100 μL were used to determine recoveries, and duplicate 100-μL quantities were used in the EIA. Testosterone concentrations were determined with a four-parameter, logistic curve-fitting program (Microplate Manager; BioRad) and corrected for incomplete recoveries.

Samples from different years were run in different assays. In 2003–2004 assays, the intraplate coefficient of variation (calculated from standard samples of known concentration), ranged from 1% to 19% (mean 9%), and interplate variation was 20%. In the 2006 assay, intraplate variation ranged from 4% to 19% (mean 12%), and interplate variation was 23%. To correct for interplate variation, we multiplied each measurement by the grand mean of standards across all plates within a given data set divided by the plate mean of standards.

Within a given year, multiple plasma samples from the same individual were analyzed on the same plate. Individuals were randomly assigned to plates, and samples within a plate were randomly assigned to wells.

Statistical Analyses

All statistical analyses were performed using SPSS 14.0 for Windows. To test for relationships between testosterone and behavior, we used restricted maximum likelihood to fit linear mixed models (Verbeke and Molenberghs 2000). Such models allow for simultaneous estimation of structured random effects (an error variance-covariance matrix) and tests of fixed effects. For the analysis of territorial aggression, we used a compound symmetrical covariance structure for the random portion of the model (SPSS 14.0 Command Syntax Reference, SPSS, Chicago). This model fits estimates of two parameters, error variance across subjects and error covariance within subjects (which is analogous to repeatability). The latter term allowed us to account for repeated testosterone measurements from an individual. In the analysis of parental behavior, we fit a diagonal covariance structure, which estimated separate error variances for the 2 years of the study (SPSS 14.0 Command Syntax Reference). Because only one individual

was sampled for parental behavior in both years, we could not estimate the within-subjects covariance term.

The relationships between behavior and initial (pre-GnRH-challenge) testosterone (natural log transformed), postchallenge testosterone (natural log transformed), and GnRH-induced testosterone increase (natural log postchallenge testosterone − natural log initial testosterone) were tested in separate analyses. In order to control for variables that may have affected them (Jawor et al. 2006), testosterone measurements were used as the dependent variables in our mixed models. We used Type I (sequential) sums of squares, which allowed us to control for these variables before testing for covariation with the behavior of interest. For both data sets, we included handling time (min, natural log transformed), day of year, and mass (g) as continuous fixed effects. For the analysis of parental behavior, year was also entered as a categorical fixed effect. Behavioral measurements were the last fixed effect entered into each model.

In order to visualize the relationships between testosterone and behavior, we calculated adjusted values of all testosterone measures. For the territorial aggression data set, we used general linear models that included handling time, day of year, and mass as well as an individual term to generate individual least squares means. For the parental care data set, we used models that included year, handling time, day of year, and mass. Adjusted values were calculated by adding the residual value for each individual to the overall mean for each testosterone value.

Because we were interested in generalized territorial aggression, and because territorial behaviors were intercorrelated, we extracted a single principal component to describe response to simulated territorial intrusions. The first principal component, which described 47% of variance, was loaded as in table 1 and was used as our measurement of aggression in the statistical analyses.

Results

Territorial Behavior

In birds for which territorial behavior was measured, mean initial testosterone (± 1 SE) was 1.85 ± 0.182 ng mL^{-1}, mean postchallenge testosterone was 7.17 ± 0.564 ng

Table 1: Loadings of the first principal component (PC1) of territorial behavior measured in simulated territorial intrusions

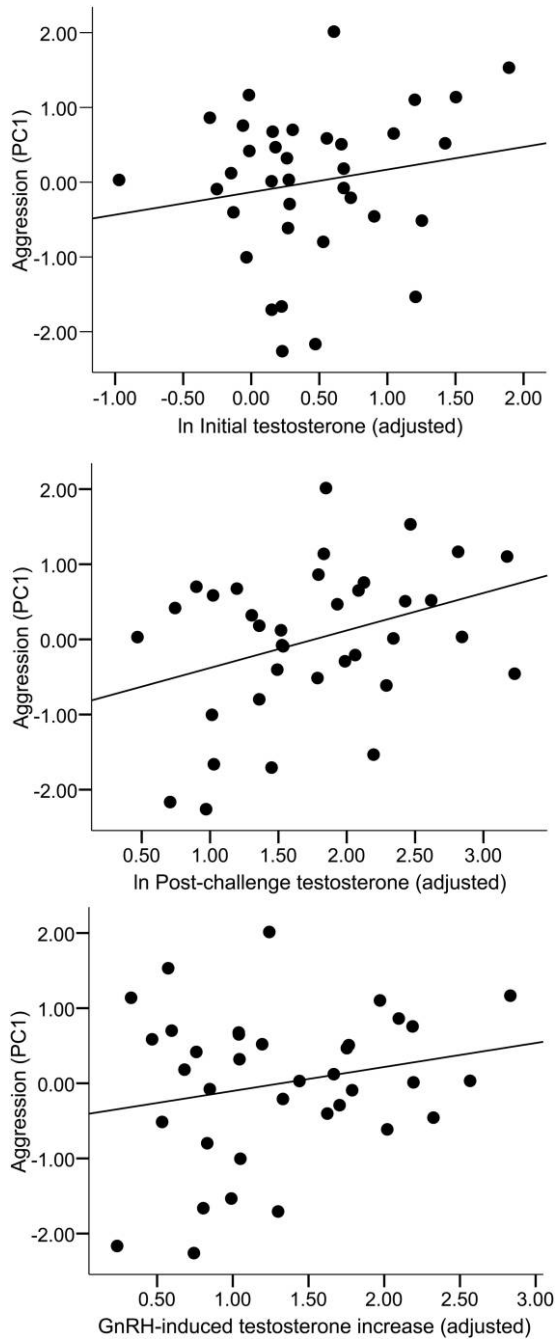| Behavior	PC1 loading
Latency	−.81
Time spent within 5 m	.74
Flyovers	.61
Songs	.53

Figure 1: Relationships between the first principal component of territorial aggression and initial testosterone levels (natural log transformed), postchallenge (gonadotropin-releasing hormone [GnRH]) testosterone levels (natural log transformed), and the magnitude of GnRH-induced increase (ln postchallenge − ln initial). Aggression was measured only once for each individual. Testosterone levels were measured either once or twice. Testosterone values were adjusted for multiple measurements as well as handling time, mass, and day of year, as described in the "Methods."

mL^{-1}, and the mean GnRH-induced increase was 5.31 ± 0.510 ng mL^{-1} ($n = 57$). The natural log–transformed values were correlated as follows: initial-post, $r = 0.51$; initial-increase, $r = -0.41$; and post-increase, $r = 0.58$ ($P \leq .002$, $n = 57$). On average, focal males responded to the simulated territorial intrusions in 115 ± 29.1 s, spent 542 ± 44.7 s within 5 m of the cage, performed 2.8 ± 0.46 flights over the cage, and sang 54 ± 6.5 songs ($n = 36$).

The first principal component of territorial behavior was positively related to postchallenge testosterone (fig. 1; table 2). In other words, males that produced higher absolute levels of testosterone in response to the GnRH challenge tended to show shorter response latency, spent more time within 5 m of the lure, performed more flyovers, and produced more songs. Initial testosterone levels showed a trend toward a relationship with aggression, but there was no significant relationship with GnRH-induced increase (fig. 1; table 2). Inspection of figure 1 suggests that this pattern may have been driven by the two individuals with the highest adjusted initial testosterone levels. These individuals were sampled only once and produced relatively low GnRH-induced increases (0.33 and 0.57), suggesting that their testosterone levels may have been elevated when they were captured.

Parental Behavior

In males that were measured for parental behavior, mean initial testosterone was 2.95 ± 0.152 ng mL^{-1}, mean postchallenge testosterone was 6.88 ± 0.591 ng mL^{-1}, and the

Table 2: Linear mixed models of relationships between testosterone and territorial aggression (first principal component)

Fixed effects	F	df	b	P
Ln initial testosterone:				
Ln handling time[a]	10.22	1, 37.6	−.45	.003
Day	.95	1, 51.9	−.01	.33
Mass	1.39	1, 23.9	.03	.25
PC1 aggression[b]	3.30	1, 24.3	.14	.082
Ln postchallenge testosterone:				
Ln handling time[a]	4.72	1, 49.3	−.21	.035
Day[a]	11.50	1, 42.2	−.03	.002
Mass	.12	1, 34.0	−.08	.73
PC1 aggression[a]	29.31	1, 29.3	.22	.033
GnRH-induced increase (ln postchallenge − ln initial):				
Ln handling time	.81	1, 52.0	.28	.37
Day[a]	5.78	1, 35.8	−.02	.022
Mass	2.72	1, 42.3	−.15	.11
PC1 aggression	.69	1, 32.6	.09	.41

Note: GnRH = gonadotropin-releasing hormone.

[a] Relationships with $P < .05$.

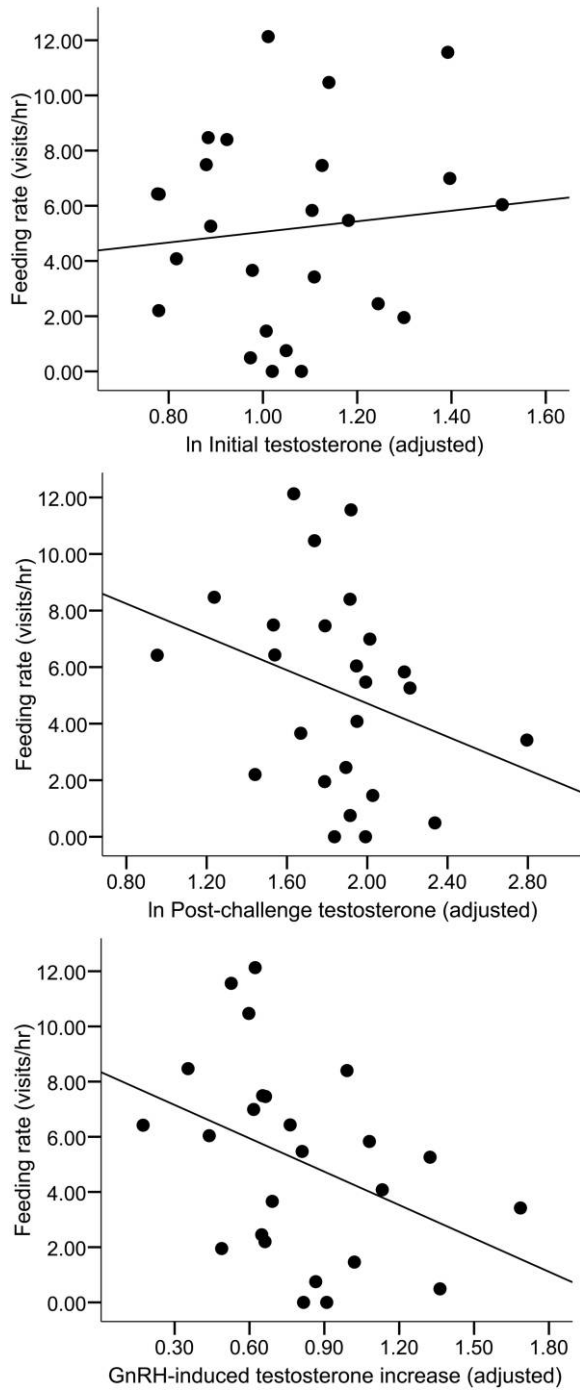[b] Relationships with $.05 < P < .1$.

Figure 2: Relationships between nestling feeding rate and initial testosterone levels (natural log transformed), postchallenge (gonadotropin-releasing hormone [GnRH]) testosterone levels (natural log transformed), and the magnitude of GnRH-induced increase (ln postchallenge − ln initial). Testosterone values were adjusted for year, handling time, mass, and day of year, as described in the "Methods."

mean GnRH-induced increase was 3.93 ± 0.564 ng mL^{-1} ($n = 25$). The natural log–transformed values were correlated as follows: initial-post, $r = 0.34$ ($P = .10$); initial-increase, $r = -0.27$ ($P = .20$); and post-increase, $r = 0.82$ ($P < .001$; $n = 25$). Mean feeding rate was 5.2 ± 0.71 visits h^{-1}, which was comparable with the feeding rate previously shown by control males in the presence of a female (Ketterson et al. 1992).

Nestling feeding rate was negatively related to the magnitude of GnRH-induced testosterone increase (fig. 2; table 3). There was a trend toward a relationship with postchallenge testosterone, and there was no significant relationship with initial testosterone (fig. 2; table 3).

The average feeding rate was higher in 2003 (6.7 visits h^{-1}) than in 2004 (3.7 visits h^{-1}, $P = .04$), but there was no year difference in any of the testosterone measures (table 3), suggesting that year differences did not generate our results. Indeed, our results did not differ if we used year-adjusted values for nestling feeding rate.

Discussion

To address natural variation in the hormonal resolution of the trade-off between mating effort and parental effort, we assessed individual variation in the responsiveness of the HPG axis and its covariation with aggressive and parental behavior. Implantation studies of free-living dark-eyed juncos have shown that experimentally enhanced tes-

Table 3: Linear mixed models of relationships between testosterone and nestling feeding rate

Fixed effects	F	df	b	P
Ln initial testosterone:				
Year	.11	1, 14.9		.75
Ln handling time	2.26	1, 14.4	−.12	.15
Day[a]	6.34	1, 15.7	−.01	.023
Mass	.16	1, 18.9	−.03	.70
Feeding rate	1.57	1, 9.9	.02	.24
Ln postchallenge testosterone:				
Year	.41	1, 16.9		.53
Ln handling time	.02	1, 14.7	−.04	.89
Day	.30	1, 14.5	−.002	.59
Mass[b]	3.23	1, 11.2	−.11	.099
Feeding rate[b]	3.43	1, 19.0	−.05	.079
GnRH-induced increase (ln postchallenge − ln initial):				
Year	.87	1, 18.2		.36
Ln handling time[b]	3.59	1, 16.7	.17	.076
Day	.001	1, 16.5	−.001	.98
Mass[b]	4.16	1, 13.0	−.12	.062
Feeding rate[a]	4.51	1, 18.7	−.05	.047

Note: GnRH = gonadotropin-releasing hormone.

[a] Relationships with $P < .05$.

[b] Relationships with $.05 < P < .1$.

tosterone can alter the resolution of this trade-off (Ketterson and Nolan 1992, 1994, 1999). Our results based on testosterone response to a GnRH challenge indicate that this conclusion can be generalized to natural differences among individuals on which selection may act. In general, the behavior of males that produced higher testosterone levels suggested higher mating effort and lower parental effort. Both territorial aggression and parental behavior were predicted by aspects of the hormonal response to stimulation of the HPG axis. Specifically, males that produced higher maximum testosterone levels in response to GnRH were more aggressive when responding to a simulated territorial intrusion, and males that showed a greater increase above initial testosterone levels after a GnRH challenge fed their offspring less often. Initial testosterone showed a trend toward a positive relationship with aggression but was not significantly related to parental behavior. These results suggest that individuals vary along a testosterone-mediated continuum between individuals that invest heavily in the survival of their offspring and those that avoid parental care to seek additional mating opportunities.

Although these relationships were measured in different individuals in different years, the common physiological link suggests that short-term testosterone elevations may underlie individual variation in the resolution of the mating effort/parental effort trade-off in this species. To the extent that this variation is heritable, the mating effort/parental effort trade-off should be able to respond to selection. Below, we discuss the implications of our findings for understanding both the mechanistic basis and the evolution of hormonally mediated trade-offs.

Short-Term Testosterone Elevation and Behavior

The "challenge hypothesis" states that testosterone should be most closely associated with behavior during periods of social instability (Wingfield et al. 1987, 1990). Such a relationship is expected to arise because testosterone levels increase in response to social stimuli, probably via stimulation of the HPG axis (Harding 1981; Wingfield 1985). One common pattern is that testosterone levels and aggression show a concomitant increase in response to simulated territorial intrusions (Wingfield 1985).

In this study, we found that the testosterone levels produced in response to a GnRH challenge (which predicts testosterone levels produced in response to a male social stimulus; J. W. McGlothlin, J. M. Jawor, T. J. Greives, J. M. Casto, J. L. Phillips, and E. D. Ketterson, unpublished manuscript) were positively correlated with the aggressive response to a territorial intruder. Because GnRH challenges and measurement of territorial aggression were separated by as much as 1 month, this result suggests that

the relationship represents a property of an individual; in other words, males probably vary consistently in both ability to produce testosterone and aggressiveness. This suggestion is supported by the repeatability of GnRH challenge response across the breeding season previously demonstrated in male juncos (Jawor et al. 2006). Although we did not measure aggressive response repeatedly in this study, response to a territorial intrusion has been shown to be repeatable in a closely related species (song sparrows *Melospiza melodia*; Nowicki et al. 2002).

We also found a trend toward a relationship between initial testosterone levels before GnRH challenge and aggressive response. Initial and postchallenge levels showed a strong positive correlation during early breeding, suggesting that the correlations between aggression and these testosterone measures may be indicating the same relationship. Alternatively, some males may have been captured after engaging in a territorial dispute or courtship and thus had elevated initial levels of testosterone. An examination of the data shows that this may be the case. The positive trend appears to be driven by the two males with the highest initial levels, which also displayed weak increases in response to the GnRH challenge, suggesting that their HPG axis may have already been maximally stimulated. A third possibility is that baseline testosterone levels and short-term elevations may both be important for producing aggressive behavior. Although aggressive response is clearly related to individual variation in the HPG system, further study is necessary to disentangle the influences of baseline testosterone levels and short-term increases.

Male parental behavior was negatively related to the testosterone response to a GnRH challenge and showed a nonsignificant trend toward a negative relationship with absolute postchallenge levels. There was no relationship between parental behavior and initial testosterone levels. Despite maintaining low initial plasma levels during nestling feeding, males retain the physiological ability to produce short-term testosterone elevations that in some cases approach breeding season peak levels (Ketterson and Nolan 1992; Jawor et al. 2006). Our results suggest that variation in the magnitude of these elevations, rather than initial testosterone levels, may underlie natural variation in parental behavior. Short-term testosterone elevation may act as a mechanism allowing a male to alternate between feeding nestlings and other behaviors such as territory defense and mate search.

If a causal relationship between short-term testosterone elevations and behavior exists, it may arise by two mechanisms that are not mutually exclusive. First, previous socially induced testosterone elevations may have had a "priming" effect on the behavior. It is well established that testosterone elevations contribute to the persistence of ter-

ritorial aggression, particularly in winners of contests (e.g., Wingfield 1994; Trainor et al. 2004). Males may respond more aggressively to an intruder because high levels of testosterone produced in response to previous interactions with competitors have had persistent effects on brain regions related to aggression. Such an effect is likely to act through regulation of gene expression, which is the classical mechanism of steroid action (Nelson 2005). The extent to which individuals vary in the physiology related to this effect is unknown. Males that produced higher levels of testosterone in response to GnRH challenges are likely to have produced larger natural testosterone increases during past encounters with conspecifics and may thus have upregulated expression of certain genes necessary for producing territorial aggression.

Second, testosterone may have rapid activational effects on behavior, likely occurring by way of mechanisms that do not involve gene expression. In an elegant experiment using toadfish, Remage-Healey and Bass (2006) demonstrated a rapid increase in calling behavior when males were fed 11-ketotestosterone, the primary androgen in fishes. Neurophysiological studies of a closely related species suggest that these behavioral changes occur due to hormonal effects on the activity of the vocal control region of the brain (Remage-Healey and Bass 2004).

Such rapid effects of testosterone on behavior may be mediated by conversion to estrogens by the enzyme aromatase at the target location. Testosterone often exerts its effects on the brain via this mechanism (Nelson 2005). There is strong evidence that estrogens may have rapid neuromodualtory effects, which may in turn cause behavioral shifts (Maggi et al. 2004; Cornil et al. 2006). For example, in Japanese quail (*Coturnix japonica*), rapid changes in sexual behavior have been linked to changes in the bioavailability of estrogen (Balthazart et al. 2006). Cornil et al. (2006) suggest that aromatization may commonly underlie rapid behavioral effects of testosterone, such as responses to territorial intruders or the production of sexual behavior. This may occur via rapid changes in circulating plasma testosterone, as seen in many songbirds, or by rapid modulation of brain aromatase activity, as demonstrated in quail (Cornil et al. 2006).

Although it is clear how short-term testosterone increases might directly mediate the expression of territorial behavior, it is less obvious how such changes might affect parental behavior. One possibility is that males respond to stimuli, such as a neighbor's song or the presence of a female, by temporarily shifting their activity from parental behavior to song or courtship. The magnitude of testosterone increases produced in response to these stimuli may affect the likelihood or duration of such a shift. In support of this hypothesis, testosterone-implanted males show increased song rate coincident with decreased parental care

(Ketterson et al. 1992). The temporal pattern of nestling feeding we observed is consistent with intermittent shifts in behavior. Visits to the nest were not evenly distributed in time (see also Clotfelter et al. 2007). Males with the lowest feeding rates (<4 visits h^{-1}) often left nestlings unattended for more than 1 h at a time (J. W. McGlothlin, personal observation). These long gaps suggest that, rather than being poor foragers, males that feed infrequently are allocating effort to other activities. Although social stimulation may cause testosterone elevations, leading to behavioral shifts, an alternative explanation is that males produce such elevations spontaneously. Further study is necessary to examine whether male or female stimuli have the capacity to alter male parental behavior.

Evolution of Testosterone-Mediated Trade-Offs

A long-term implantation study conducted in this population found that, on average, males treated with testosterone had higher fitness than controls (Reed et al. 2006). This effect occurred because testosterone-treated males had higher mating success (as measured by extrapair fertilizations), which more than compensated for their decreased survival (Raouf et al. 1997; Reed et al. 2006). Such results suggest that selection should favor males with constitutively elevated testosterone.

However, our data suggest an alternative option. Levels of testosterone produced in response to GnRH challenge are similar to those produced both at the early breeding season peak (late March–early April) and by treatment with testosterone implants (Ketterson et al. 1992; Jawor et al. 2006). This shows that males can produce short-term increases in testosterone as needed to support territorial (and perhaps sexual) behavior, without maintaining circulating testosterone at a constitutively high level. Males may thus avoid or moderate some of the costs of high testosterone without losing the ability to produce testosterone-mediated behavior (Wingfield et al. 2001). In the long term, males with flexible HPG axes would likely be favored over males with inflexible, but high, testosterone levels.

The potential costs of producing short-term testosterone increases have not been explored. To the extent that the costs of testosterone are related to the production of testosterone-mediated behavior (rather than systemic effects such as immunosuppression), short-term elevation may indeed be costly. One of the common results of implantation studies is that experimentally enhanced testosterone leads to increased activity (e.g., Lynn et al. 2000). This effect is likely to be associated with short-term testosterone elevation as well, as evidenced by the association with territorial aggression shown here. Increased activity may be beneficial in terms of mating success, leading to more

vigorous territorial defense or the ability to encounter more females (Chandler et al. 1994; Raouf et al. 1997) while at the same time imposing survival costs such as depletion of energy stores or increasing visibility to predators (Ketterson et al. 1991; Reed et al. 2006). Future studies should examine in more detail the relationship of natural levels of testosterone and trade-offs involving survival.

Although selection pressures on baseline testosterone levels may indeed differ, it is not clear whether these aspects of the HPG system are likely to evolve separately. In this study, initial and postchallenge testosterone levels were positively correlated, which reflects that they are two manifestations of a common hormonal system. The maintenance of circulating levels of testosterone as well as the production of short-term increases depends on the stimulation of the HPG axis, suggesting that common genes are likely to be associated with variation in both. The evolutionary independence of different aspects of the HPG system may depend on whether behavioral effects depend more on absolute or relative levels of testosterone. The results presented here, although not conclusive, suggest that both may be important.

Although our results indicate that natural variation in testosterone levels is associated with behavioral variation, we do not wish to diminish the importance of other aspects of the hormonal system. Our GnRH challenge protocol was designed to assess variation in pituitary and gonadal response, but important variation is likely to exist both upstream and downstream of the HPG axis. In order for GnRH to be released, individuals must integrate environmental and social stimuli in the neural pathways that stimulate the hypothalamus. Individuals are likely to vary in sensory as well as neural mechanisms. Downstream, testosterone is often converted to another hormone, and regardless of whether conversion occurs, hormones must interact with receptors to have an effect. Variation likely exists in enzyme activity, receptor expression, and the pathways activated by the hormone-receptor complex. Important evolutionary changes may occur at any of the steps along this complex pathway (Hau 2007).

Conclusion

We have demonstrated that individual variation in parental and territorial behavior is related to individual variation in testosterone production. Furthermore, our results suggest that the ability to produce short-term testosterone increases may be more important for the mediation of this trade-off than circulating baseline levels, at least in *Junco hyemalis carolinensis*. This is not likely to be true in all species, however. For example, some species do not decrease parental care when testosterone is elevated (Hunt et al. 1999; Van Duyse et al. 2000, 2002; Lynn et al. 2002,

2005), and some do not increase testosterone in response to social stimuli (reviewed in Landys et al. 2007). Interestingly, these species differences seem to be related to changes in the mating effort/parental effort trade-off. Behavioral insensitivity to testosterone seems to occur in species where male parental care is critical to offspring survival (Lynn et al. 2005). Further, species that rear only a single brood (and thus may have less conflict between periods of mating effort and parental effort) are less likely to show socially modulated testosterone increases (Landys et al. 2007). Across species, testosterone-mediated trade-offs seem to be evolutionarily labile, responding to changes in social and environmental selection pressures (Hau 2007). Our study provides initial evidence that the testosterone-mediated trade-off between mating effort and parental effort varies among individuals, and thus, the raw material necessary for selection to generate among-species patterns seems to exist within populations.

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