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Seasonal and individual variation in response to GnRH challenge in male dark-eyed juncos (*Junco hyemalis*)

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Abstract

Concentrations of gonadal steroids such as testosterone (T) often vary widely in natural populations, but the causes and particularly the consistency of this variation is relatively unexplored. In breeding males of a wild population of the dark-eyed junco (*Junco hyemalis*), we investigated seasonal and individual variation in circulating T during two breeding seasons by measuring the responsiveness of the HPG axis to a standardized injection of gonadotropin-releasing hormone (GnRH). Individuals were bled prior to and 30 min after injection. Pre- and post-challenge levels of T were measured using EIA. Many subjects were sampled repeatedly across multiple breeding stages. Plasma T concentrations nearly doubled in response to GnRH during early spring, but showed significantly smaller increases in later breeding stages. When controlling for seasonal variation in response to challenge, we also found repeatable differences among individuals, indicating individual consistency in the release of T in response to a standardized stimulus. These seasonal and individual differences may arise from comparable variation in responsiveness of the pituitary or a decline in gonadal sensitivity to downstream gonadotropins. In contrast, pre-challenge T showed almost no seasonal changes and did not differ consistently among individuals. To our knowledge, this is the first demonstration of individual repeatability of short-term hormonal changes in a wild population. Such repeatability suggests that hormonal plasticity might evolve in response to changing selection pressures.

Keywords: GnRH challenge; Gonadotropin releasing hormone; Dark-eyed junco; *Junco hyemalis*; Testosterone; Seasonality

1. Introduction

Gonadotropin-releasing hormone (GnRH) stimulates the release of luteinizing hormone (LH) from the pituitary, and in males, LH stimulates the production of testosterone (T) by the testes. In some species, the pituitary has been shown to respond similarly to GnRH both during the breeding season and outside of it (Wingfield et al., 1979), suggesting that the fluctuations in T observed over the course of the year in many species are influenced by something other than the pituitary itself. In male birds, reports indicate seasonal differences in concentrations of GnRH in the brain (reviewed in Ball and Hahn, 1997; Dawson et al., 2001; Sharp et al., 1998) and in the size and number of cells that produce GnRH (Bentley et al., 2000a,b; Cho et al., 1998; Deviche et al., 2000; Foster et al., 1987; Marsh et al., 2002; Saldanha et al., 1994; Stevenson and MacDougall-Shackleton, 2005; but see Meddle et al., 2006 for a photoperiodic species, and Bentley et al., 2003a; MacDougall-Shackleton et al., 2001 for species that are not strictly photoperiodic). Seasonal variation in GnRH concentration has been linked to the initiation of gonadal recrudescence, seasonal variation in the concentration of circulating T, and the development of photorefractoriness (Ball and Hahn, 1997; Dawson and Goldsmith, 1997; Foster et al., 1987; Marsh et al., 2002; Pereyra et al., 2005).

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GnRH challenges are often used to assess the reproductive condition of individuals (Goymann and Wingfield, 2004; Hirschenhauser et al., 2000; Lacombe et al., 1991; Millesi et al., 2002; Moore et al., 2002; Schoech et al., 1996; Soma and Wingfield, 2001; Wingfield et al., 1979, 1991). In a typical challenge, GnRH is administered either intravenously or intramuscularly, and concentrations of LH and/or T prior to and after the challenge are compared. This method has allowed researchers to compare GnRH response among classes of individuals and populations and to relate the level of response to reproductive activities, age, and, social status.

Quantifying seasonal and individual variation in hormonal mechanisms is important for understanding plasticity and evolution of these mechanisms and their associated behaviors (Adkins-Regan, 2005). GnRH challenges may be used to examine seasonal and individual variation in the responsiveness of the hypothalamic–pituitary–gonadal (HPG) axis, and understanding such variation may be important for at least two reasons. First, concentrations of T produced by GnRH challenge represent a response to a standardized stimulus and thus are more representative of individual variation than differences in endogenous T. Second, short-term increases in T, similar to those produced by a GnRH challenge, may be produced during male–male and male–female interactions, and may underlie expression of many T-mediated behaviors (Moore, 1983; Pinxten et al., 2003; Wingfield, 1985; Wingfield et al., 1994, 2001). Such short-term responses may allow individuals to increase T when necessary without paying the costs of constitutively elevated T (Wingfield et al., 2001). Quantifying variation in short-term changes of T may lead to a greater understanding of variation in social behaviors and how such behaviors and their underlying mechanisms may respond to selection.

Male dark-eyed juncos (*Junco hyemalis*) have seasonally variable T, with peak levels occurring during territory establishment (late March and early April in a population residing in eastern North America; Ketterson and Nolan, 1992; Ketterson et al., 2005). T declines in mid-April and remains low while males assist in parental care (Ketterson and Nolan, 1992; Ketterson et al., 2005). Yearling males have lower peak concentrations of T than older males, but this age difference disappears after the seasonal peak (Corbit and Deviche, 2005; Deviche et al., 2000; Ketterson and Nolan, 1992). After the conclusion of breeding, juncos undergo pre-basic molt (August to mid-December, Nolan et al., 2002) during which T is virtually undetectable (Ketterson and Nolan, 1992; Ketterson et al., 2005; Nolan et al., 1992, 2002). As is the case in a number of other Emberizids (e.g., Wingfield, 1985), males are able to produce short-term increases in T during simulated territory intrusions that are similar in magnitude to the maximum early-breeding season baseline (McGlothlin et al., in preparation). However, the extent to which the ability to produce these short-term increases varies across the breeding season and among individuals is unknown.

In this study, we repeatedly challenged individual male juncos with GnRH at multiple time points during two successive breeding seasons, allowing us to investigate both seasonal and individual variation in the responsiveness of the HPG axis in reproductive individuals. We predicted that T response in male juncos to a GnRH challenge would remain constant during the breeding season. We also predicted that individual response would be variable and repeatable. Given previously described age-related differences in peak plasma T (Deviche et al., 2000; Ketterson et al., 1992) we also investigated whether age influenced the magnitude of T response.

2. Methods

2.1. Captive GnRH challenges

We performed pilot trials on captive juncos to test the effectiveness of intramuscular GnRH challenges (see Millesi et al., 2002, for this technique in mammals) and the time course of the T response. On 12 July 2002, GnRH challenges were performed on 10 captive male juncos at Kent Farm Bird Observatory in Bloomington, IN. All birds were in breeding condition (visible cloacal protuberances) and had no detectable molt. Before administration of the GnRH challenge, an initial blood sample (~50 μl) was collected from the alar wing vein using heparinized microcapillary tubes. Birds were then assigned randomly to receive either one injection of 1.25 μg of chicken GnRH-I (Sigma L0637, Moore et al., 2002) dissolved in 50 μl of 0.1 M phosphate-buffered saline solution (PBS) in the left pectoralis major (hereafter low dose, $n = 5$) or one such injection in each pectoralis major (for a total dose of 2.5 μg in 100 μl, hereafter high dose, $n = 5$) using a Hamilton syringe. Following the injection, additional blood samples (~50 μl) were collected from each bird at 3–4 of 6 time points: 30 min, 1 h, 2 h, 3 h, 4 h, and 6 h post-injection. Three birds from each of the two dosage treatments were sampled at each time point. Between sample collections birds were housed individually indoors in small cages. Plasma from all blood samples was stored at −20 °C until assayed. Assays were performed using the enzyme immunoassay (EIA) protocol detailed below.

2.2. Field GnRH challenges

Over the breeding seasons of 2003 and 2004, 90 adult male juncos were captured near Mountain Lake Biological Station (MLBS) (37°22′N, 80°32′W) in Giles Co., Virginia, USA. Upon capture, each bird was returned to the central lab at MLBS in a holding bag. There, each bird was fitted with an aluminum United States Fish & Wildlife Service leg band and a unique combination of plastic color bands if the bird had not already been banded. We obtained standard morphometric measurements (flattened wing chord length, tail length, tarsus [all in mm], mass [g]) and determined the age class of the bird (yearling or older adult [⩾2 years]) using the colors of the primary wing coverts and iris (Nolan et al., 2002). Older adults were later assigned an age in years using their initial appearance in the capture records of previous years. All birds that were first banded as pre-adults or yearlings could be assigned an exact age. If a bird was an older adult when it was first captured, we made the conservative assumption that it was 2 years old.

After banding and morphometric measurements, we collected a blood sample from each individual (~100 μl) and then administered a GnRH injection immediately afterwards. Based on our pilot data we chose to perform GnRH challenges using the lower dose (1.25 μg cGnRH in 50μl of PBS, see Section 3). We collected a second blood sample (~100 μl) at exactly 30 min post-challenge. Birds were placed in holding bags between sample collections. Following GnRH challenges birds were released back at the site of capture. Blood samples (both pre- and post-challenge samples) were centrifuged and the plasma fraction drawn off and stored at −20 °C until assayed.

We attempted to challenge males repeatedly, up to four times in a given year, during four sampling periods classified as Early Breeding A, Early Breeding B, Nesting, and Late Breeding. Birds captured for the first time in the spring belonged to the Early Breeding A category (2003: 28 April–16 May, $n = 53$; 2004: 21 April–11 May, $n = 46$, combined $n = 99$). Early Breeding A birds that were recaptured and sampled a second time during the early spring were classified as Early Breeding B (2003: 6 May–16 May, $n = 26$; 2004: 1 May–15 May, $n = 11$, combined $n = 37$). Early Breeding B challenges were performed 7–21 days after Early Breeding A challenges (mean = 10.4 days). During Early Breeding (A and B), many birds were beginning to nest, but the exact stage of reproduction was unknown for most of them (dates of first egg were 26 April in 2003 and 25 April in 2004). Some birds were captured a third time and given a GnRH challenge while feeding 6- to 7-day-old nestlings; these were classified as Nesting (2003: 25 May–29 June, $n = 14$, 2004: 20 May–20 July, $n = 14$). A final set of birds was captured at the end of the breeding season, but prior to the onset of molt, and these were classified as Late Breeding (2003: 15 July–6 August, $n = 7$; 2004: 20 July–5 August, $n = 9$). All sampling periods occurred after the typical early-breeding season maximal T peak in juncos (26 March–14 April, Ketterson and Nolan, 1992). Overall 5 individuals were challenged a total of 5 times, 6 were challenged 4 times, 12 were challenged 3 times, 28 were challenged 2 times, and 39 were challenged once. Twenty-three (23) individuals received challenges in both 2003 and 2004, 35 were challenged in 2003 only, and 32 were challenged in 2004 only.

During the early breeding stages, individuals were captured at random using baited mist nets and Potter traps. During the Nesting stage we attempted to passively capture males with a mist net as they approached the nest; however, a few birds were captured using conspecific song playback (5 in 2003; no statistical differences in initial or post-challenge T when compared to birds captured passively). During Late Breeding, males were again captured randomly using baited mist nets.

2.3. Testosterone assays

We determined T using an EIA kit (Assay Designs, Inc., #901-065) (described in Clotfelter et al., 2004). For the analysis of samples approximately 2000 cpm of H^3-T were added to allow calculation of recoveries after extraction (2 extractions with diethyl ether). Extracts were re-suspended in 50 µl of 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. T concentrations were determined with a 4-parameter logistic curve-fitting program (Microplate Manager; Bio-Rad Laboratories, Inc.) and corrected for incomplete recoveries. Intra-assay variation, which was calculated as the coefficient of variation of values obtained from standard samples of known concentration, ranged from 1 to 19%, inter-assay variation was 19.7%. Inter-assay variation was increased due the use of plates from multiple kit lots. For each individual all plasma samples were analyzed in the same assay and were randomly assigned to wells on the plate.

2.4. LH assay

To confirm the effect of the intramuscular GnRH injection on pituitary output, we measured LH in 29 males from the 2003 and 2004 breeding seasons. The plasma came from pre- and post-challenge samples collected during Early Breeding A (no additional blood collected, analysis focused on birds whose plasma samples from this period were sufficient for both the T and the LH assays). Approximately 30 µl of plasma was used in an RIA using the homologous chicken LH radioimmunoassay (RIA) (Follett et al., 1972). All samples were run in duplicate in a single assay. Intra-assay coefficient of variation was 4.2% and the detection limit was 0.039 ng/ml.

2.5. Statistical analyses

To ask whether the GnRH challenge was effective in elevating T, we used paired-samples t-tests for each breeding stage (Early Breeding A and B, Nesting, Late Breeding) to test for a difference between initial and post-challenge T. We used a repeated-measures linear mixed model with restricted maximum likelihood estimation to analyze variation in initial T and post-challenge T. Such a model accounts for repeated measures on the same individual while allowing for unbalanced sampling across time points (Verbeke and Molenberghs, 2000). Each model included breeding stage and year as fixed factors, a stage × year interaction, age in years, mass, and natural-log transformed handling time (in min, defined as the time elapsed between removing a bird from the mist net or Potter trap and the beginning of the first blood sample) as covariates, and individual identity as a random repeated effect. Results did not differ if age class (yearling or older adult) was substituted for age in years, so only the latter is reported. Because we expected T concentrations before and after GnRH challenge to be correlated, we included initial T as a covariate in the post-challenge T mixed model. This allowed us to test for differences in the magnitude of increase above initial T while holding variation in initial T constant. A compound symmetrical covariance structure was used for the repeated measures. This structure assumes constant variance across measures and constant covariance between measures. The covariance between measures was used to calculate the within-individual correlation coefficient, a measure of repeatability (Lessells and Boag, 1987). To explore seasonal effects further, when appropriate we compared estimated marginal means for each breeding stage using t-tests. In order to correct for multiple tests, we used a Bernoulli equation to calculate the probability of finding multiple significant differences by chance (Moran, 2003).

3. Results

3.1. Captive GnRH challenges

Results of the preliminary study on captives showed that the GnRH challenge led to increases in T (repeated measures linear mixed model with first-order autoregressive error structure, time $F_{6,23.2} = 15.84$, $p < 0.0001$, Fig. 1). T was highest at 30 min post-challenge ($p < 0.0001$) and remained elevated above initial levels at 1 h ($p = 0.003$), although levels at 1 h were significantly lower than at 30 min post-challenge ($p = 0.005$). By 2 h, T had returned to initial levels ($p = 0.562$). At 3 and 4 h, T was slightly, but not significantly, lower than initial levels ($p = 0.07$, 0.08), and at 6 h, T was indistinguishable from initial levels ($p = 0.50$). Results did not differ based on dosage ($F_{1,11.5} = 1.97$, $p = 0.19$) and there was no dosage × time interaction ($F_{6,23.2} = 0.87$, $p = 0.54$), although T in the low-dose treatment did not

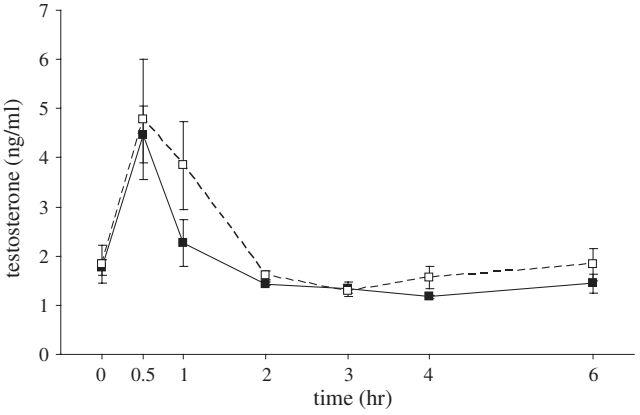

Fig. 1. Time course of response to GnRH challenge in 10 captive male juncos. Males received either a low dose (1.25 µg, open squares/dotted line) or a high dose (2.5 µg, closed squares/solid line).

decrease as rapidly post-challenge as did T in the high-dose treatment (Fig. 1). These data suggest that intramuscular GnRH challenges produce a peak in T that has begun to decline by 1 h post-challenge, and that our low dose was sufficient to induce maximal T steroidogenesis.

3.2. LH levels

Post-challenge levels of LH were greater than initial levels (mean initial LH, 1.71 ng/ml ±0.14 ng/ml, mean post-challenge LH, 2.39 ng/ml ±0.21 ng/ml; paired Samples *t*-test; $t = 4.005$, df = 28, $P < 0.0001$), indicating that intramuscular GnRH challenge increased circulating LH levels. Initial and post-challenge levels of LH were correlated (Pearson Correlation; $r = 0.59$, $P = 0.001$, $n = 29$). However, initial and post-challenge concentrations of T did not co-vary with initial or post-challenge LH concentrations (all r's < 0.23, all P's > 0.24).

3.3. Response to GnRH challenge

The GnRH challenge was effective in elevating T in each breeding stage (all t's ⩾ 3.89, all p's ⩽ 0.001, Fig. 2). Post-challenge T was positively correlated with initial T (Table 1, Fig. 3). For Early Breeding A mean initial T was 3.36 ng/ml (range 1.81–11.94 ng/ml) and mean post-challenge T was 8.23 ng/ml (range 2.41–24.81 ng/ml). For Early Breeding B mean initial T was 3.26 ng/ml (range 1.83–7.56 ng/ml) and mean post-challenge T was 6.40 ng/ml (range 3.09–11.96 ng/ml). For Nesting, mean initial T was 2.94 ng/ml (range 1.96–5.367 ng/ml) and mean post-challenge T was 6.81 ng/ml (range 2.74–17.47 ng/ml). Last, for Late Breeding mean initial T was 3.25 ng/ml (range 2.30–6.77 ng/ml) and mean post-challenge T was 7.30 ng/ml (range 2.30–17.02 ng/ml).

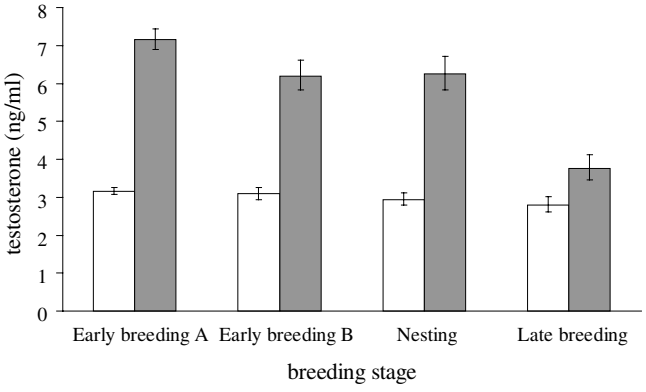

Fig. 2. Seasonal variation in initial (open bars) and post-GnRH challenge testosterone (shaded bars). Plotted data are back-transformed estimated marginal means ± 1 SE from the analyses in Table 1. See Section 3 for mean testosterone and observed maximum/minimum T.

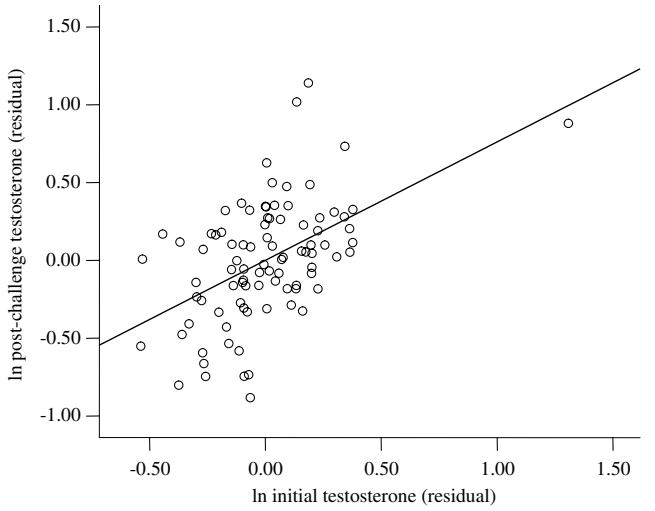

Fig. 3. Relationship between initial and post-GnRH challenge testosterone (for all collection points, Early Breeding A and B, Nesting, and Late Breeding). Each data point represents the estimated marginal mean value for each individual, controlling for other variables in the model.

Table 1
Linear mixed model analysis of post-GnRH challenge in plasma testosterone concentrations

Random effects		Estimate	Wald Z	p
Repeatability		0.36	3.32	0.001
Residual variance component		0.13	8.28	<0.0001
Fixed effects	**Estimate**	**df**	**F**	**p**
Stage		3, 123.3	17.34	<0.0001
Year		1, 155.0	1.43	0.23
Stage × Year		3, 117.8	1.09	0.36
Age	−0.019	1, 97.9	0.86	0.36
Mass	−0.110	1, 165.8	22.77	<0.0001
ln capture time	−0.070	1, 144.6	2.91	0.09
ln initial testosterone	0.842	1, 156.5	79.40	<0.0001

3.4. Seasonal variation

Initial T did not vary significantly among breeding stages ($F_{3,153.1} = 1.14$, $p = 0.33$, Fig. 2) or between years ($F_{1,168.9} = 2.35$, $p = 0.13$), and there was no year × breeding stage interaction ($F_{3,149.8} = 0.53$, $p = 0.66$). When we examined seasonal variation with a continuous effect of Julian calendar date (instead of breeding stage, thus using a finer time scale), both pre- and post-challenge T showed a significant decline as the season progressed (pre-challenge T $F_{1,155.3} = 6.85$, $b = -0.002$, $p = 0.01$; post-challenge T $F_{1,131.8} = 53.74$, $b = -0.007$, $p < 0.0001$).

The magnitude of the increase in T above initial levels following the GnRH challenge differed among breeding stages (Table 1, Fig. 2). When we compared the estimated marginal mean increases in each breeding stage, we found that increases during Early Breeding A were higher than during the other breeding stages (Early Breeding B, $p = 0.030$; Nesting $p = 0.073$; Late Breeding $p < 0.0001$, Fig. 1). Early Breeding B and Nesting increases were also higher than those in Late Breeding ($p < 0.0001$, Fig. 2). However, Early Breeding B and Nesting increases did not differ from each other ($p = 0.93$). The probability of obtaining 4 significant results out of 6 tests at $\alpha = 0.05$ is 0.0001 (Bernoulli equation; Moran, 2003), allowing us to retain the

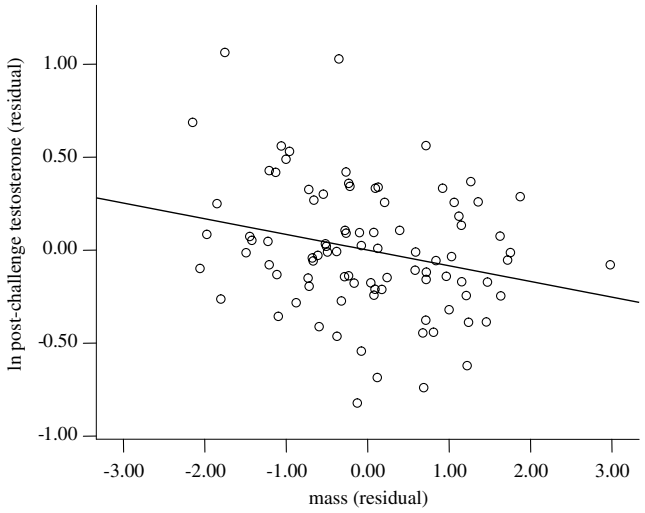

Fig. 4. Relationship between mass and post-GnRH challenge testosterone (for all collection points, Early Breeding A and B, Nesting, and Late Breeding). Each data point represents the estimated marginal mean value for each individual, controlling for other variables in the model.

significance of these findings. There was no year effect or year × breeding stage interaction (Table 1).

3.5. Effects of age, mass, and handling time

There were no significant effects of age or mass on initial T ($F \leqslant 0.005$, $p \geqslant 0.94$). Male age had no effect on the response to the GnRH challenge (Table 1). Heavier males showed a smaller increase in T above initial levels (Table 1, Fig. 4). Both initial ($\beta = -0.063$, $F_{1,169.0} = 3.63$, $p = 0.059$) and post-challenge T (Table 1) showed marginally non-significant decreases with increased handling time.

3.6. Individual repeatability

When controlling for other variables using a linear mixed model, the increase in T above initial levels was significantly repeatable (Table 1). Initial T showed low, non-significant repeatability ($r = 0.11$, Wald $Z = 1.17$, $p = 0.24$).

4. Discussion

We found that breeding male dark-eyed juncos show significant seasonal and individual variation in the increase in T following a GnRH challenge. Specifically, the response to challenge decreased as the breeding season progressed, falling first early in the breeding season coincident with the onset of nesting and again later in the breeding season as nesting activity slowed. Age did not influence the level of response to GnRH challenge. Response was associated with mass, with lighter individuals showing a higher response overall. Response decreased slightly with increased handling time, but the effect was not statistically significant. We also found that individual response to a GnRH challenge was repeatable; birds that were strong responders to GnRH in one sampling period also responded strongly at other times. Despite the decrease in response as breeding progressed, the repeatability of the response indicates that individual differences in short-term hormone increase remain consistent.

4.1. Seasonal variation in response to GnRH challenge

The cause of the decrease in response to GnRH challenge over the course of the breeding season is not known and may stem from variation in the number or specificity of receptors for GnRH in the pituitary, receptors for LH on the testes, or the ability of Leydig cells to respond to LH challenge. When pituitary response to GnRH, measured as LH output, has been investigated, most studies in breeding and non-breeding birds and mammals have seen no variation in pituitary response (Kriegsfeld et al., 1999; Spinks et al., 2000; Wingfield et al., 1979; but see Gardiner et al., 1999). We found that LH increased following an intramuscular injection of GnRH, but did not assess LH outside of the early breeding season and will have to await future studies to determine whether the seasonal decline in T response to GnRH reflects seasonal decline in the sensitivity of the pituitary to GnRH.

When changes in T, as opposed to LH, have been measured in response to GnRH, the comparisons have typically involved breeding and non-breeding animals, and smaller responses were observed in non-breeding animals (Hirschenhauser et al., 2000; Millesi et al., 2002). This cross-seasonal reduction in response has been clearly linked to gonadal regression during the non-breeding season. Based on these prior studies, we did not expect to find a decrease in T response over the course of the breeding season. Although we did not measure gonad size, all males in this study were in reproductive condition (cloacal protuberance evident) during Early Breeding A and B, and Nesting. We did not include post-reproductive individuals (indicated by molt) in our Late Breeding sample.

Although we are not able to address the source of seasonal decline in T in response to GnRH observed in this study, several studies have now found that a regulatory neuropeptide, gonadotropin inhibitory hormone (GnIH), varies in concentration with season in the avian brain (Bentley et al., 2003b), is influenced by changes in melatonin (Ubuka et al., 2005), and leads to decreased LH (Bentley et al., 2003b; Ubuka et al., 2006; Yin et al., 2005). However, studies have not addressed whether GnIH is upregulated in the brain to influence GnRH secretion, pituitary response to GnRH, or testes response to LH. Further work will be needed to integrate our findings from natural populations with those conducted in the laboratory.

From a functional perspective, a seasonal decline in T response to GnRH may be favored by selection in biparental birds because it facilitates a shift from mating effort (e.g., territory acquisition and courtship) to parental effort (e.g., nestling feeding) as nesting progresses (Wingfield et al., 1990). A seasonal decrease in T response to GnRH may allow males to respond less strongly to territorial intrusions

and potential extra-pair mates, thus avoiding a shift of activity away from paternal care.

4.2. Age and response to GnRH challenge

In previous studies, older male juncos are socially dominant (Ketterson et al., 1992) and have been shown to have higher levels of T than younger males, particularly early in the breeding season (Deviche et al., 2000; Ketterson and Nolan, 1992; Ketterson et al., 1992, 2001). Because of these factors we predicted that they would have a stronger T response to GnRH. However, in this study, age had no effect on either initial T or response to GnRH challenge. The lack of an age effect in our study is likely due to the timing of our sampling. The primary age difference occurs only in peak seasonal T, which typically occurs earlier than the initiation of this study (Ketterson et al., 1992, 2001). It is possible that if GnRH challenges are administered during territory acquisition, there may be an influence of age on post-challenge levels. An alternative hypothesis takes into account differing social pressures experienced by older and younger males. Older males may acquire territories and mates earlier in the season than younger males and may hold onto these resources more readily than younger males. If older males are settled on territories early, and due to their social dominance not easily removed from these territories, then selection may favor a rapid reduction in response to GnRH inducing stimuli in older males soon after territory/mate acquisition.

4.3. The effects of mass and handling time on GnRH challenge response

The effect of mass on the response to GnRH challenges in which heavier males produced a smaller response may have occurred due to dosage. Because our pilot data indicated that two very different doses of GnRH led to similar peak responses, we did not adjust the dose of GnRH given to birds of varying mass as was done in some other studies (Millesi et al., 2002). However, because of the intramuscular method of administration, diffusion of GnRH in males with more muscle mass may have taken slightly longer, giving rise to the observed effect. Such an effect of diffusion may have led to a later peak in circulating T. Indeed, birds captured at all time points had very little visible fat, thus differences in mass are largely attributable to differences in muscle mass as opposed to fat stores. Examination of such an effect would require measuring the GnRH challenge response at a finer time scale. Another, not mutually exclusive, possibility is that a greater ability to produce T may have led to increased activity and therefore to a decrease in mass. Male juncos with experimentally enhanced T are more physically active and show increased weight loss during early spring but not later (Ketterson et al., 1991).

Levels of T can be reduced in response to stressors (Sapolsky et al., 2000), such as handling during capture prior to the challenge. We found that handling time had a negative effect on initial and post-challenge levels of T. However, this effect was weak and non-significant, and it is unlikely that it influenced our findings.

4.4. Repeatability of response to GnRH challenges

The individual consistency in response to GnRH challenges is an important result from an evolutionary perspective. Although species and populations clearly differ in hormonal mechanisms of behavior, we know little about how natural selection acts on hormones and how strong evolutionary responses to selection may be (Adkins-Regan, 2005). Our results suggest that although levels of T may vary both seasonally and on a shorter timescale, individual differences remain consistent. Such consistency is necessary for an evolutionary response to selection because repeatability sets an upper bound for heritability, the percentage of phenotypic variance that may be inherited by the next generation (Lynch and Walsh, 1998). Results from domestic animals indicate that GnRH challenge response may respond to artificial selection (Haley et al., 1990; McNeilly et al., 1988; Robinson et al., 1994), but no studies have yet measured its heritability or response to selection in natural populations.

Our findings may be of relevance to those interested in predicting the timing of reproduction and response to climate change (e.g., Nussey et al., 2005). To the extent that repeatability predicts heritability (Lynch and Walsh, 1998), and response to GnRH predicts readiness to initiate breeding, it may be possible to use challenges to compare populations for potential to show an evolutionary response to climate change. If the repeatability and heritability of GnRH response is high, evolutionary response to changing climate is likely to be rapid.

4.5. Comments on methodology

The method of intramuscular administration of GnRH is relatively new (Millesi et al., 2002), with intravenous administration representing the more standard method. As such, some considerations must be taken into account. First, greater concentrations of GnRH are needed. There are several points where GnRH may be lost, either due to protein degradation prior to it reaching the pituitary, or in delays in GnRH diffusing from the muscles to the circulatory system. In our pilot study, we tested two doses of GnRH and found no variation in level of response in captive juncos and chose to use a single dosage. Given that lighter birds had a stronger response to GnRH it is conceivable that these individuals were experiencing a higher effective dose than heavier birds. Using a single dose may not be suitable for larger species, or individuals with large fat stores. With perhaps minor methodological adjustments this method of GnRH administration may allow for an easier method of conducting GnRH challenges under field conditions.

5. Conclusions

In summary, this study provides evidence that T response to GnRH challenge varies among individuals and over time, but is repeatable within individuals. The gradual decline of the T response in individuals, and the population as a whole, suggests plasticity that may lead to adaptive changes in behavior. We suggest that intramuscular GnRH challenges should be used in future studies to assess individual ability to produce T and its relationships to behavior. Similar studies in other species should be conducted to determine whether individual consistency of T production capability is a common phenomenon. Last, the observed seasonal decrease in responsiveness of the population as a whole suggests that some downstream factor, in addition to the available levels of GnRH in the brain, may regulate seasonal differences in T production.

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References

Adkins-Regan, E., 2005. Hormones and Animal Social Behavior. Princeton University Press, Princeton.

Ball, G.F., Hahn, T.P., 1997. GnRH neuronal systems in birds and their relation to the control of seasonal reproduction. In: Parhar, I.S., Sakuma, Y. (Eds.), GnRH Neurons: Gene to Behavior. Brain Shuppan Publishers, Tokyo.

Bentley, G.E., Dawson, A., Goldsmith, A.R., 2000a. Lack of gonadotropin-releasing hormone neuron response to decreasing photoperiod in thyroidectomized male starlings (*Sturnus vulgaris*). J. Exper. Zool. 287, 74–79.

Bentley, G.E., Spar, B.D., MacDougall-Shackleton, S.A., Hahn, T.P., Ball, G.F., 2000b. Photoperiodic regulation of the reproductive axis in male zebra finches, *Taeniopygia guttata*. Gen. Comp. Endocrinol. 117, 449–455.

Bentley, G.E., Audage, N.C., Hanspal, E.K., Ball, G.F., Hahn, T.P., 2003a. Photoperiodic response of the hypothalamo–pituitary–gonad axis in male and female canaries, *Serinus canaria*. J. Exp. Zool. 296A, 143–151.

Bentley, G.E., Perfito, N., Ukena, K., Tsutsui, K., Wingfield, J.C., 2003b. Gonadotropin-inhibitory peptide in song sparrows (*Melospiza melodia*) in different reproductive conditions, and in house sparrows (*Passer domesticus*) relative to chicken-gonadotropin-releasing hormone. J. Neuroendocrinol. 15, 794–802.

Cho, R.N., Hahn, T.P., MacDougall-Shackleton, S., Ball, G.F., 1998. Seasonal variation in brain GnRH in free-living breeding and photorefractory house finches (*Carpodacus mexicanus*). Gen. Comp. Endocrin. 109, 244–250.

Clotfelter, E.D., O'Neal, D.M., Gaudioso, J.M., Casto, J.M., Parker-Renga, I.M., Snajdr, E.A., Duffy, D.L., Nolan Jr., V., Ketterson, E.D., 2004. Consequences of elevating plasma testosterone in females of a socially monogamous songbird: evidence of constraints on male evolution? Horm. Behav. 46, 171–178.

Corbit, C., Deviche, P., 2005. Age-related difference in size of brain regions for song learning in adult male dark-eyed juncos (*Junco hyemalis*). Brain Behav. Evol. 65, 268–277.

Dawson, A., Goldsmith, A.R., 1997. Changes in gonadotropin-releasing hormone (GnRH-I) in the pre-optic area and median eminence of starlings (*Sturnus vulgaris*) during the recovery of photosensitivity and during photostimulation. J. Reprod. Fertility 111, 1–6.

Dawson, A., King, V.M., Bentley, G.E., Ball, G.F., 2001. Photoperiodic control of seasonality in birds. J. Biol. Rhythms 16, 365–380.

Deviche, P., Saldanha, C.J., Silver, R., 2000. Changes in brain gonadotropin-releasing hormone- and vasoactive intestinal polypeptide-like immunoreactivity accompanying reestablishment of photosensitivity in male dark-eyed juncos (*Junco hyemalis*). Gen. Comp. Endocrinol. 117, 8–19.

Follett, B.K., Scanes, C.G., Cunningham, F.J., 1972. A radioimmunoassay for avian luteinizing hormone. J. Endocrinol. 52, 359–378.

Foster, R.G., Plowman, G., Goldsmith, A.R., Follett, B.K., 1987. Immunohistochemical demonstration of marked changes in the LHRH system of photosensitive and photorefractory European starlings (*Sturnus vulgaris*). J. Endocrinol. 115, 211–220.

Gardiner, K.J., Boyd, I.L., Follett, B.K., Racey, P.A., Reijnders, P.J.H., 1999. Changes in pituitary, ovarian, and testicular activity in harbour seals (*Phoca vitulina*) in relation to season and sexual maturity. Can. J. Zool. 77, 211–221.

Goymann, W., Wingfield, J.C., 2004. Competing females and caring males. Sex steroids in African black coucals, *Centropus grillii*. Anim. Behav. 68, 533–540.

Haley, C.S., Lee, G.J., Ritchie, M., Land, R.B., 1990. Direct responses in males and correlated responses for reproduction in females to selection for testicular size adjusted for body weight in young male lambs. J. Reprod. Fertil. 89, 383–396.

Hirschenhauser, K., Möstl, E., Péczely, P., Wallner, B., Dittami, J., Kotrschal, K., 2000. Seasonal relationships between plasma and fecal testosterone in response to GnRH in domestic ganders. Gen. Comp. Endocrinol. 118, 262–272.

Ketterson, E.D., Nolan Jr., V., 1992. Hormones and life histories-an integrative approach. Am. Nat. 140, S33–S62.

Ketterson, E.D., Nolan Jr., V., Sandell, M., 2005. Testosterone in females: mediator of adaptive traits, constraint on sexual dimorphism, or both? Am. Nat. 166, S85–S98.

Ketterson, E.D., Nolan Jr., V., Wolf, L., Ziegenfus, C., 1992. Testosterone and avian life histories: effects of experimentally elevated testosterone on behavior and correlates of fitness in the dark-eyed junco (*Junco hyemalis*). Am. Nat. 140, 980–999.

Ketterson, E.D., Nolan Jr., V., Wolf, L., Ziegenfus, C., Dufty, A.M., Ball, G.F., Johnsen, T.S., 1991. Testosterone and avian life histories: the effect of experimentally elevated testosterone on corticosterone and body mass in dark-eyed juncos. Horm. Behav. 25, 489–503.

Ketterson, E.D., Nolan Jr., V., Casto, J.M., Buerkle, C.A., Clotfelter, E., Grindstaff, J.L., Jones, K.J., Lipar, L.L., McNabb, F.M.A., Neudorf, D.L., Parker-Renga, I., Schoech, S.J., Snajdr, E., 2001. Testosterone, phenotype and fitness a research program in evolutionary behavioral endocrinology. In: Dawson, A., Chaturvedi, C.M. (Eds.), Avian Endocrinology. Narosa Publishing House, New Delhi.

Kriegsfeld, L.J., Drazen, D.L., Nelson, R.J., 1999. Effects of photoperiod and reproductive responsiveness on pituitary sensitivity to GnRH in male prairie voles (*Microtus ochrogaster*). Gen. Comp. Endocrinol. 116, 221–228.

Lacombe, D., Cyr, A., Matton, P., 1991. Plasma LH and androgen levels in the red-winged blackbird (*Agelaius phoeniceus*) treated with a potent GnRH analogue. Comp. Biochem. Physiol. 99A, 603–607.

Lessells, C.M., Boag, P.T., 1987. Unrepeatable repeatabilities—a common mistake. Auk 104, 116–121.

Lynch, M., Walsh, B., 1998. Genetics and Analysis of Quantitative Traits. Sinauer Assoc., Sunderland.

MacDougall-Shackleton, S.A., Deviche, P.J., Crain, R.D., Ball, G.F., Hahn, T.P., 2001. Seasonal changes in brain GnRH immunoreactivity and song-control nuclei volumes in an opportunistically breeding songbird. Brain Behav. Evol. 58, 38–48.

Marsh, R.H., MacDougall-Shackleton, S.A., Hahn, T.P., 2002. Photorefractoriness and seasonal changes in the brain in response to changes in day length in American goldfinches (*Carduelis tristis*). Can. J. Zool. 80, 2100–2107.

McGlothlin, J.W., Jawor, J.M., Greives, T.J., Casto, J.M., Phillips, J.L., Ketterson, E.D. Hormonal responses reinforce honesty of sexual ornamentation in a songbird, in preparation.

McNeilly, J.R., Fordynce, M., Land, R.B., Martin, G.B., Sringbett, A.J., Webb, R., 1988. Changes in the feedback control of gonadotrophin secretion in ewes from lines selected for testis size in the ram lamb. J. Reprod. Fertil. 84, 213–221.

Meddle, S.L., Wingfield, J.C., Millar, R.P., Deviche, P.J., 2006. Hypothalamic GnRH-I and its precursor during photorefractoriness onset in free-living male dark-eyed juncos (*Junco hyemalis*) of different year classes. Gen. Comp. Endocrinol. 145, 148–156.

Millesi, E., Hoffmann, I.E., Steurer, S., Metwaly, M., Dittami, J.P., 2002. Vernal changes in the behavior and endocrine responses to GnRH application in male European ground squirrels. Horm. Behav. 41, 51–58.

Moore, I.T., Perfito, N., Wada, H., Sperry, T.S., Wingfield, J.C., 2002. Latitudinal variation in plasma testosterone levels in birds of the genus *Zonotrichia*. Gen. Comp. Endocrinol. 129, 13–19.

Moore, M.C., 1983. Effect of female sexual displays on the endocrine physiology and behavior of male white-crowned sparrows, *Zonotrichia leucophrys*. J. Zool. 199, 131–148.

Moran, M.D., 2003. Arguments for rejecting the sequential Bonferroni in ecological studies. Oikos 100, 403–405.

Nolan Jr., V., Ketterson, E.D., Ziegenfus, C., Cullen, D.P., Chandler, C.R., 1992. Testosterone and avian life histories: effects of experimentally elevated testosterone on prebasic molt and survival in male dark-eyed juncos. Condor 94, 364–370.

Nolan, Jr., V., Ketterson, E.D., Cristol, D.A., Rogers, C.M., Clotfelter, E.D., Titus, R.C., Schoech, S.J., Snajdr, E., 2002. Dark-eyed Junco (*Junco hyemalis*). In: Poole, A., Gill, F. (Eds.), The Birds of North America, No. 716. The Birds of North America, Philadelphia, PA.

Nussey, D.H., Postma, E., Gienapp, P., Visser, M.E., 2005. Selection on heritable phenotypic plasticity in a wild bird population. Science 310, 304–306.

Pereyra, M.E., Sharbaugh, S.M., Hahn, T.P., 2005. Interspecific variation in photo-induced GnRH plasticity among nomadic Cardueline finches. Brain Behav. Evol. 66, 35–49.

Pinxten, R., DeRidder, E., Eens, M., 2003. Female presence affects male behavior and testosterone levels in the European starling (*Sternus vulgaris*). Horm. Behav. 44, 103–109.

Robinson, O.W., Lubritz, D., Johnson, B., 1994. Realized heritability estimates in boars divergently selected for testosterone levels. J. Anim. Breed. Genet. 111, 35–42.

Saldanha, C.J., Deviche, P.J., Silver, R., 1994. Increased VIP and decreased GnRH expression in photorefractory dark-eyed juncos (*Junco hyemalis*). Gen. Comp. Endocrinol. 93, 128–136.

Sapolsky, R.M., Romero, L.M., Munck, A.U., 2000. How do glucocorticoids influence stress responses? Integrating permissive, suppressive, stimulatory, and preparative actions. Endocrine Rev. 21, 55–89.

Schoech, S.J., Mumme, R.L., Wingfield, J.C., 1996. Delayed breeding in the cooperatively breeding Florida scrub-jay (*Aphelocoma coerulescens*): inhibition or the absence of stimulation? Behav. Ecol. Sociobiol. 39, 77–90.

Sharp, P.J., Dawson, A., Lea, R.W., 1998. Control of luteinizing hormone and prolactin secretion in birds. Comp. Biochem. Physiol. C 119, 275–282.

Soma, K.K., Wingfield, J.C., 2001. Dehydroepiandrosterone in songbird plasma: seasonal regulation and relationship to territorial aggression. Gen. Comp. Endocrinol. 123, 144–155.

Spinks, A.C., Bennett, N.C., Faulkes, C.G., Jarvis, J.U.M., 2000. Circulating LH levels and the response to exogenous GnRH in the common mole-rat: Implications for reproductive regulation in this social, seasonal breeding species. Horm. Behav. 37, 221–228.

Stevenson, T.J., MacDougall-Shackleton, S.A., 2005. Season- and age-related variation in neural cGnRH-I and cGnRH-II immunoreactivity in house sparrows (*Passer domesticus*). Gen. Comp. Endocrinol. 143, 33–39.

Ubuka, T., Bentley, G.E., Ukena, K., Wingfield, J.C., Tsutsui, K., 2005. Melatonin induces the expression of gonadotropin-inhibitory hormone in the avian brain. Proc. Natl. Acad. Sci. USA 102, 3052–3057.

Ubuka, T., Ukena, K., Sharp, P.J., Bentley, G.E., Tsutsui, K., 2006. Gonadotropin-inhibitory hormone inhibits gonadal development and maintenance by decreasing gonadotropin synthesis and release. Endocrinology, 147, 1187–1194.

Verbeke, G., Molenberghs, G., 2000. Linear Mixed Models for Longitudinal Data. Springer-Verlag, New York.

Wingfield, J.C., 1985. Short term changes in plasma levels of hormones during establishment and defence of a breeding territory in male song sparrows, *Melospiza melodia*. Horm. Behav. 19, 174–187.

Wingfield, J.C., Hegner, R.E., Lewis, D.M., 1991. Circulating levels of luteinizing hormone and steroid hormones in relation to social status in the cooperatively breeding white-browed sparrow weaver, *Ploce-passer mahali*. J. Zool., Lond. 225, 43–58.

Wingfield, J.C., Whaling, C.S., Marler, P.R., 1994. Communication in vertebrate aggression and reproduction: the role of hormones. In: Knobil, E., Neill, J.E. (Eds.), Physiology of Reproduction, 2nd ed. Raven Press, New York.

Wingfield, J.C., Lynn, S.E., Soma, K.K., 2001. Avoiding the 'costs' of testosterone: ecological bases of hormone–behavior interactions. Brain Behav. Evol. 57, 239–251.

Wingfield, J.C., Crim, J.W., Mattocks Jr., P.W., Farner, D.S., 1979. Responses of photosensitive and photorefractory male white-crowned sparrows (*Zonotrichia leucophrys gambelii*) to synthetic mammalian luteinizing hormone releasing hormone (Syn-LHRH). Biol. Reprod. 21, 801–806.

Wingfield, J.C., Hegner, R.E., Dufty, A.M., Ball Jr., G.F., 1990. The "challenge hypothesis": theoretical implications for patterns of testosterone secretion, mating systems, and breeding systems. Am. Nat. 136, 829–846.

Yin, H., Ukena, K., Ubuka, T., Tsutsui, K., 2005. A novel G protein-coupled receptor for gonadotropin-inhibitory hormone in the Japanese quail (*Coturnix japonica*): identification, expression and binding activity. J. Endocrinol. 184, 257–266.