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Testosterone response to GnRH in a female songbird varies with stage of reproduction: implications for adult behaviour and maternal effects

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Summary

1. Despite considerable recent interest in plasma and yolk testosterone (T) in female birds, relatively little is known about environmental regulation of female T, individual variation in female T or the relationship between plasma and yolk T.

2. In breeding females of a wild population of dark-eyed junco (*Junco hyemalis*), we assessed variation in the responsiveness of the hypothalamo-pituitary-gonadal (HPG) axis to a challenge with gonadotropin-releasing hormone (GnRH) by measuring circulating T before and 30 min after a standardized injection of GnRH. We asked whether response to challenge varied seasonally or with stage of reproduction and whether it was repeatable within individuals or related to T deposited in eggs.

3. Initial and post-challenge levels of T were measured using enzyme immunoassay. In a subset of these females, luteinising hormone (LH) was measured using radioimmunoassay (RIA). In addition, eggs were collected from nests of 15 females that had received a GnRH challenge, and yolk T was measured using RIA.

4. During most of the breeding season, plasma T did not increase in response to GnRH. GnRH consistently caused increases in plasma T only during the 7 days before oviposition, when females were rapidly depositing yolk in eggs but had not yet begun to lay them. Among a small subset of females we found a positive correlation between the magnitude of this increase in plasma T in response to GnRH during egg development and the amount of T deposited in the yolk of eggs collected at a later time.

5. These results suggest that ovarian response to GnRH-induced increases in LH is greatest when females are actively depositing yolk into eggs. Factors that stimulate the release of GnRH during egg formation may result in higher levels of plasma T which could influence adult female behaviour. Further, because plasma T was correlated with later yolk T, factors that stimulate GnRH release may also lead to higher levels of yolk T potentially influencing offspring development or behaviour.

Key-words: testosterone, aggressive behaviour, female birds, maternal effects, seasonality, GnRH challenge

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Introduction

The effects of testosterone (T) on morphology, behaviour and physiology have been studied extensively in male birds (Wingfield *et al.* 1990; Wingfield & Hahn 1994; Saino, Møller & Bolzern 1995; Hasselquist *et al.* 1999; Ketterson & Nolan 1999; Kimball & Ligon 1999; Peters *et al.* 2000; Casto, Nolan & Ketterson 2001; Stoehr & Hill 2001; Wingfield, Lynn & Soma 2001; Strasser & Schwabl 2004; Day, McBroom & Schlinger 2006). Recently, the role of T produced by females has received more attention (Elekonich & Wingfield 2000; Hau *et al.* 2000; Langmore, Cockrem & Candy 2002; Clotfelter *et al.* 2004; Groothuis & von Engelhardt 2005; Jawor,

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Young & Ketterson 2006a). T in female birds often varies seasonally, with the highest levels at the beginning of the breeding season and near-undetectable levels in the non-breeding season (reviewed in Ketterson, Nolan & Sandell 2005). Female T also varies with social system; it is higher in colonial than in solitarily nesting species and higher in species with monogamous mating systems than in species with non-monogamous systems (Ketterson *et al.* 2005). In a number of studies, T has been shown to positively influence female aggression or to increase during intrasexual interactions (Hegner & Wingfield 1987; Langmore *et al.* 2002; Smith *et al.* 2005; Zysling *et al.* 2006), while in others, female T has been shown to be unresponsive to intrasexual competitors (Schwabl *et al.* 1988; Elekonich & Wingfield 2000; Hau *et al.* 2000; Jawor *et al.* 2006a). Because of these mixed findings, the relationship between T and female behaviour merits further study.

In addition to its potential behavioural effects in adult females, T may mediate maternal effects when it is deposited in the egg yolk (Schwabl 1993, 1996a; Lipar & Ketterson 2000; Sockman & Schwabl 2000; Gil 2003; Groothuis & von Engelhardt 2005). Elevations in yolk T have been found to have both advantageous and detrimental effects on offspring behaviour, development and fitness (reviewed in Gil 2003; Groothuis & von Engelhardt 2005). In some studies, experimental elevation of circulating T in adult females has led to increased concentrations of egg yolk T (Hackl *et al.* 2003; Clotfelter *et al.* 2004; Rutkowska *et al.* 2005). Inter- or intrasexual interactions have been shown to lead to an increase in yolk T in a number of species (Schwabl 1996b, 1997; Whittingham & Schwabl 2002; Groothuis & Schwabl 2002; Mazuc *et al.* 2003; Pilz & Smith, 2004; Navara *et al.* 2006). In most of these studies, it was not known whether plasma T had increased as well (but see Navara *et al.* 2006). Though both plasma T and yolk T can increase as a result of social interactions, potential links between adult behaviour, circulating plasma T and yolk T are not well understood.

In this study, we explored natural variation in T levels in female dark-eyed juncos (*Junco hyemalis*) as a first step toward a better understanding of these links. We measured seasonal and individual variation in T using repeated challenges with gonadotropin-releasing hormone (GnRH). Injection of GnRH, a hypothalamic peptide, temporarily stimulates the hypothalamo-pituitary-gonadal (HPG) axis, leading to release of luteinising hormone (LH) from the pituitary, and in turn, steroid hormones from the gonads. In females, LH stimulates androgen synthesis by the theca interna cells of the ovarian follicle (Johnson 2000). These androgens may be then be aromatized to oestradiol, transported to the granulosa layer of the follicle, or released into the bloodstream without further modification, where they may be delivered to target tissues, including the brain. In a typical GnRH 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 most often been used to assess the reproductive condition of individuals (Wingfield *et al.* 1979; Lacombe, Cyr & Matton 1991; Wingfield, Hegner & Lewis 1991; Schoech, Mumme & Wingfield 1996; Hirschenhauser *et al.* 2000; Soma & Wingfield 2001; Millesi *et al.* 2002; Moore *et al.* 2002; Goymann & Wingfield 2004; Spinney, Bentley & Hau 2006).

In a recent study, we found that GnRH challenges consistently produced transient increases of plasma T in male juncos (Jawor *et al.* 2006b). The magnitude of these elevations decreased as the breeding season progressed, but individual males showed repeatable responses to the challenge. Further, the extent of the short-term increases in T produced by GnRH challenges was found to be correlated with socially induced increases in T brought on by bouts of territorial behaviour (J.W. McGlothlin, unpublished), suggesting that GnRH challenges can be used to assess the magnitude of natural, socially induced hormonal responses as well.

In this study, we used repeated GnRH challenges of breeding female Juncos to examine variation in both initial circulating levels and the potential to produce short-term T elevations. Although previous studies in which females were challenged with GnRH have not found significant increases in T (Wingfield *et al.* 1991; Goymann & Wingfield 2004; Spinney *et al.* 2006), our study is the first to assess variation across multiple stages of the breeding season. Sampling females at different stages allows for a finer-scaled investigation of whether and when the ovary responds to GnRH/LH with an increase in T, which may affect our understanding of variation in female T in nature (reviewed in Ketterson *et al.* 2005). In addition, we collected eggs from a subset of the females in our sample and measured the amount of T deposited in the yolk. We compared these values to the results of GnRH challenges, in order to assess possible links between variation in HPG sensitivity and deposition of T in the yolk.

Materials and methods

FIELD METHODS

Over the breeding seasons of 2003 and 2004, 71 adult female juncos were captured using mist nets and Potter traps near Mountain Lake Biological Station (MLBS) (37°22′N, 80°32′W) in Giles Co., Virginia, USA (see Jawor *et al.* 2006b for capture details). Upon capture, each bird was returned to the central laboratory at MLBS in a holding bag, where it was banded, measured and aged as described in Jawor *et al.* (2006b). Females were examined for the presence of a brood patch and their mass (g) recorded (mean female mass 23·2 g, SE = 0·197, $n = 114$).

An initial blood sample was collected from each individual (*c.* 100 μL) and then an intramuscular GnRH injection was administered immediately afterwards (time between capture and first blood collection, range 4–

135 min, mean 42·97 ± 2·22 min, $n = 114$, see below for statistical corrections in hormone levels for handling time). Based on data from a previous experiment, described in Jawor *et al.* (2006b), GnRH challenges were performed using 1·25 μg cGnRH-I in 50 μL of phosphate buffered saline (PBS). A second blood sample (*c.* 100 μL) was collected exactly 30 min post-challenge. Birds were kept in holding bags between sample collections. Following GnRH challenges, birds were released at the site of capture. Blood samples (both pre- and post-challenge samples) were centrifuged, and the plasma fraction was collected and stored at –20 °C until assayed.

Females were challenged repeatedly (maximum of two times in 2003, possible maximum of four times in 2004) during four seasonal categories. Birds captured for the first time in the spring belonged to the Early Breeding A category (dates of initial capture in 2003: 28 April to 15 May, $n = 37$; in 2004: 21 April to 10 May, $n = 33$, combined $n = 70$). Early Breeding A birds that were recaptured and sampled a second time during the early spring (which we defined as dates prior to 20 May in both years) were classified in the Early Breeding B category (2003: 8 May to 15 May, $n = 12$; 2004: 5 May to 18 May, $n = 10$, combined $n = 22$). Early Breeding B challenges were performed 7–16 days after Early Breeding A challenges (mean = 8·4 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 at the time of capture (dates of first egg within the population were 26 April in 2003 and 25 April in 2004). In 2004, some individuals were captured and challenged during a third sampling stage when they were caring for 6–7 day old nestlings, these birds were categorized as Nestling Feeding (14 May to 9 July, $n = 17$). A final set of birds was captured at the end of the breeding season, but prior to the onset of moult, and these were categorized as Late Breeding (2004: 20 July to 29 July, $n = 5$). Across all sampling periods no individuals were sampled four times, seven individuals were challenged three times, 29 individuals were challenged twice, and 35 individuals were challenged once.

Throughout the breeding season (late April to late July), nests of females on our study site were located and monitored. For a subset of the GnRH-challenge samples ($n = 63$, from 41 different females), we were able to use information about nests to determine the stage of reproduction of females on the day of GnRH challenge (see Nolan *et al.* 2002 for a detailed account of the breeding biology of Juncos). Plasma samples were classified into five categories. Those that were collected more than a week before the date of a female's first egg of the season (9–39 days before laying, mean = 23 days), or in one case, 2 weeks before the first nest-building activity was observed, were classified as Pre-nesting ($n = 12$). Samples collected within the week prior to the date of first egg (1–7 days, mean = 5 days) were classified as Egg Development ($n = 14$). The Egg Development period corresponds to a phase of rapid yolk deposition in eggs that will soon be ovulated (Johnson 2000). All but one of the females in this category had nests that were found during nest building or laying (and thus the date of first egg could be directly recorded), or nests in which the laying dates could be back-calculated from the hatching date (incubation period 12 d, Nolan *et al.* 2002). The nest of the remaining female was not found, but she was observed carrying deer hair on the day she was challenged. Females add deer hair as the last step of nest construction, and the first egg is most often laid 2–5 days after the nest is complete (Nolan *et al.* 2002). Samples collected after the first egg was laid but before the last egg of the clutch was laid were classified as Laying ($n = 4$). Incubation samples ($n = 16$) were collected during the 12-day incubation period that follows egg laying. Finally, Nestling Feeding samples ($n = 17$) were collected (as described above) when females were known to be caring for 6–7 day old nestlings.

EGG COLLECTION

When a nest was found before egg laying, or early in the laying sequence, it was visited each day and each new egg was marked so that laying order could be determined. The third egg laid was collected 24–48 h after laying in order to measure T deposited in the yolk. In our population, clutches range in size from three to five eggs with four-egg clutches being the most frequent (Nolan *et al.* 2002). All clutches from which we collected eggs were four egg clutches. There was no difference in egg yolk T based on when eggs were collected ($t_{13} = 0·98$, $P = 0·35$) and no embryonic development or yolk diffusion was evident at the time of yolk T analysis (described below). After collection, eggs were frozen at –20 °C until yolks could be assayed. Ten third-eggs were collected in 2003 and five were collected in 2004. All eggs were collected from different females ($n = 15$), and all collections took place between 7 May and 2 July (both years).

TESTOSTERONE ASSAYS

Plasma T was measured using an EIA kit (Assay Designs, Inc. (Ann Arbor, Michigan, USA), #901–065) (described in Clotfelter *et al.* 2004). Approximately 2000 cpm of ^{3}H-T were added to each sample (30 μL of plasma) to allow calculation of recoveries after extraction (two 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; BioRad Laboratories, Inc., Hercules, California, USA) 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, and therefore each measurement was multiplied by a plate correction factor prior to statistical analysis. This value was equal to the grand mean of the standard samples divided by the plate mean of the standards. All plasma samples from a given individual were analysed in the same assay and were randomly assigned to wells on the plate.

EGG YOLK ASSAY

Egg yolks were assayed for T in a single radioimmunoassay (RIA) using methods described in Lipar *et al.* (1999). Recoveries for T averaged 58%. Intra-assay variation, which was calculated as a coefficient of variation of values obtained from standard samples of known concentration, was 6·14%.

LH ASSAY

To confirm the effect of the intramuscular GnRH injection on pituitary output, LH was measured in eight samples (seven different females) from the 2003 and 2004 breeding seasons. LH was measured opportunistically when extra plasma was available following T assays. Six samples were from Early Breeding A, one was from Early Breeding B, and one was from Nestling Feeding. Approximately 30–40 μL of plasma were used. All samples were run in duplicate (15–20 μL) in a single assay, using the homologous chicken LH RIA (Follett, Scanes & Cunningham 1972). The intra-assay coefficient of variation was 4·2%, and the detection limit was 0·039 ng mL^{-1}.

STATISTICAL ANALYSES

To assess when females respond to GnRH with an increase in T, we analysed the data in two sets. First, all individuals were analysed with respect to general breeding stage (e.g. Early Breeding A, Early Breeding B, Nestling Feeding or Late Breeding). Following this, a subset of individuals was analysed with respect to their individual stages of reproduction (e.g. Pre-nesting, Egg Development, Laying, Incubation or Nestling Feeding). To test whether GnRH challenges were effective in elevating T levels, we performed paired *t*-tests that compared initial plasma T to post-challenge plasma T (both ln transformed) within sampling stages and reproductive stages. To analyse individual variation in initial and post-challenge plasma T, repeated-measures linear mixed models were used. To test for broad-scale seasonal patterns, sampling stage and year (and an interaction) were included as fixed factors, age (year), mass (g) and ln transformed handling time (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) were included as covariates, and individual identity was included as a random repeated effect. Initial plasma T (ln transformed) was included as a covariate in the model analysing post-challenge T. This allowed us to analyse the extent of the response to GnRH challenges above initial levels. A compound symmetrical covariance structure was used for the repeated measures in order to calculate the within-individual correlation coefficient, a measure of repeatability (Lessells & Boag 1987). To test for effects of stage of reproduction, we used the same models, but substituted reproductive stage for sampling stage.

Yolk T (ln transformed) was compared to ln initial plasma T, ln post-challenge plasma T and T response to GnRH challenge (ln post-challenge plasma T – ln initial plasma T) using Pearson correlations. For females challenged more than once, we used the GnRH challenge that was performed as near to, but not after, the day eggs were collected from a female's nest. In all cases, this corresponded to a female's Early Breeding A challenge. Details of the samples used in these analyses are shown in Table 1. We do not think exposure to a GnRH challenge would itself influence yolk steroid levels as the yolk is accumulated over many days and the response to the challenge is quite brief. But to test whether this affected our results we also examined correlations on a reduced data set for which te GnRH challenge data and the egg used in the comparison came from different clutches ($n = 4$; see Table 1).

Table 1. Sampling details of eggs collected from females that received GnRH challenges. Samples are listed in order of increasing response to GnRH challenge (i.e. in the order they appear in Fig. 3, left to right)

Year	Stage of reproduction when GnRH challenge performed	Days between GnRH challenge and egg collection
2004	Incubation	22
2004	Pre-nesting	39
2003	Unknown	21
2003	Incubation	24
2003	Unknown	24
2004	Unknown	34
2004	Unknown	34
2004	Pre-nesting	25
2003	Egg development	23
2003	Egg development	9
2003	Egg development	18
2003	Egg development	50
2003	Egg development	5
2003	Laying	53
2003	Egg development	42

Results

LH ANALYSIS

Levels of LH increased following GnRH challenge (Mean ± SE: initial LH 2·79 ± 0·71 ng mL^{-1}, post-GnRH challenge LH 4·39 ± 1·18 ng mL^{-1}; paired samples *t*-test; $t_7 = 2{\cdot}77$, $P < 0{\cdot}0001$), and levels of LH pre- and post-GnRH challenge were correlated within individuals (Pearson correlation; $r_6 = 0{\cdot}93$, $P = 0{\cdot}001$).

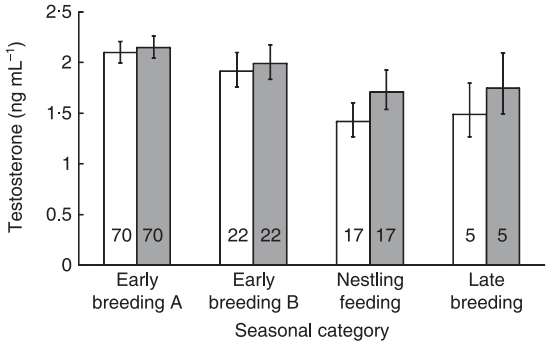

Fig. 1. Plasma T levels in adult female dark-eyed juncos, before (open bars) and after (filled bars) GnRH challenge, based on when females were challenged. Values for each stage are back-transformed estimated marginal means (± SE) from the linear mixed models described in the text. To facilitate visualization of pre- and post-challenge differences, initial T was excluded as a covariate when calculating the marginal means for post-GnRH T levels.

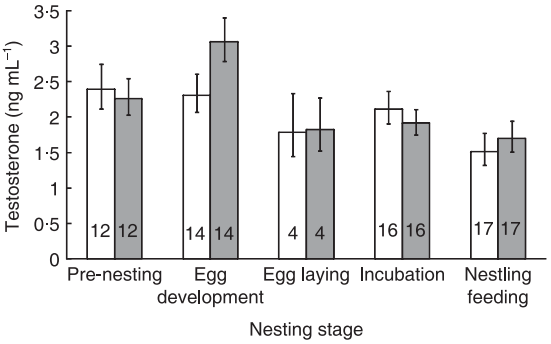

Fig. 2. Plasma T levels in adult female dark-eyed juncos, before (open bars) and after (filled bars) GnRH challenge, based on the stage of reproduction at which females were challenged. These females represent the subset of individuals from Fig. 1 that could be assigned to exact stages within the reproductive cycle. Values for each stage are back-transformed estimated marginal means (± SE) from the linear mixed models described in the text. To facilitate visualization of pre- and post-challenge differences, initial T was excluded as a covariate when calculating marginal means for post-GnRH T levels.

SEASONAL PATTERNS OF GnRH-CHALLENGE RESPONSE

Across seasonal categories, mean initial T (± SE) was 2·09 ± 0·09 ng mL^{-1} (range 0·55–5·56 ng mL^{-1}). Mean post-challenge T was 2·20 ± 0·09 ng mL^{-1} (range 0·46–6·74 ng mL^{-1}).

Initial T showed a marginally non-significant decrease as the breeding season progressed (sampling stage, $F_{3,72\cdot8} = 2\cdot68$, $P = 0\cdot053$, Fig. 1). There was a significant negative effect of mass on initial T ($b = -0\cdot05$, $F_{1,103\cdot1} = 4\cdot31$, $P = 0\cdot04$), although this effect disappears when controlling for reproductive stage and may be influenced by egg development (see below). None of the other fixed factors or covariates had a significant effect ($P \geq 0\cdot16$), and individual variation in initial T was not significantly repeatable ($r = 0\cdot22$, Wald $Z = 0\cdot91$, $P = 0\cdot36$).

Post-challenge T after GnRH injection did not differ significantly from initial T in any of the seasonal categories (Fig. 1), although there was a non-significant trend toward a response in Early Breeding A (paired t-test, $P = 0\cdot08$, all other $P > 0\cdot27$). Post-challenge T was positively correlated with initial T ($b = 0\cdot74$, $F_{1,103\cdot9} = 140\cdot23$, $P < 0\cdot0001$) and mass ($b = 0\cdot05$, $F_{1,101\cdot3} = 10\cdot40$, $P = 0\cdot002$). There were no significant differences among seasonal categories ($F_{3,92\cdot5} = 0\cdot64$, $P = 0\cdot59$), no significant repeatability ($r = 0\cdot17$, Wald $Z = 1\cdot1$, $P = 0\cdot25$), and no other significant effects ($P \geq 0\cdot22$).

REPRODUCTIVE STAGE AND GnRH-CHALLENGE RESPONSE

Initial T tended to be higher in earlier reproductive stages, although this difference was not statistically significant (Fig. 2, linear mixed model, $F_{4,43\cdot7} = 1\cdot90$, $P = 0\cdot13$). None of the other fixed effects in the model were statistically significant ($P \geq 0\cdot25$), and repeatability of initial T did not differ significantly from zero ($r = 0\cdot33$, Wald $Z = 1\cdot39$, $P = 0\cdot17$). GnRH challenge response differed significantly among reproductive stages (Fig. 2, Table 2). This difference occurred because plasma T increased in response to GnRH injection only during the Egg Development stage (Fig. 2, paired t-test, $t_{13} = 3\cdot54$, $P = 0\cdot004$, all other $P \geq 0\cdot22$). Tests of other effects on response to GnRH challenge are shown in Table 2.

Table 2. Linear mixed model analysis of post-GnRH challenge plasma testosterone concentrations, measured in females of known stage of reproduction (Pre-Breeding, Egg Development, Laying, Incubation, Nestling Feeding, see Fig. 2)

Random effects		Estimate	Wald Z	P
Repeatability		0·20	0·94	0·35
Residual variance component		0·04	4·92	< 0·0001
Fixed effects	**Estimate**	***df***	***F***	***P***
Stage		4, 47·0	7·03	0·0002
Year		1, 47·0	2·05	0·16
Stage × year		3, 48·1	1·49	0·23
Age	0·026	1, 28·3	1·00	0·33
Mass	0·029	1, 49·1	1·62	0·21
ln handling time	−0·036	1, 50·0	0·57	0·46
ln initial testosterone	0·691	1, 49·6	89·7	< 0·0001

GnRH CHALLENGE RESPONSE AND YOLK T

T concentration in yolk varied from 7·3 to 25·7 pg ng^{-1} (mean 17·1 pg ng^{-1}, $n = 15$). When examining all data, yolk T was not significantly correlated with initial plasma T, post-challenge plasma T, or the GnRH-induced change in plasma T ($r_{13} \leq 0\cdot15$, $P \geq 0\cdot52$). However, because GnRH challenges reliably increased plasma T only in birds that were developing eggs, we subdivided the data by reproductive stage. Six of the eggs we collected were laid by females that were challenged during the Egg Development stage at some time during the season, and there was a strong positive correlation between GnRH response and T deposited in the yolks of collected eggs ($r_4 = 0\cdot97$, $P = 0\cdot002$, Fig. 3). This

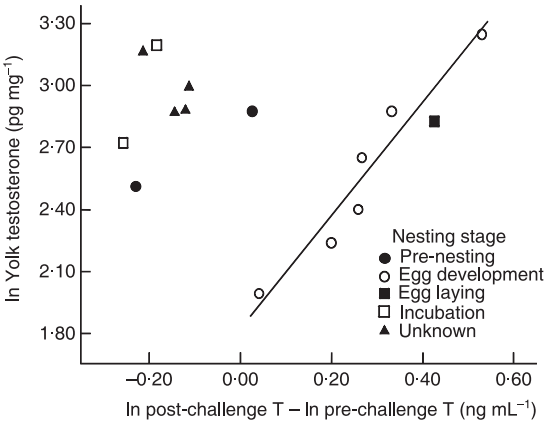

Fig. 3. Relationship between T response to GnRH challenge in different stages of reproduction (see figure key) and yolk T. Line of best fit is calculated using only Egg Development data points.

correlation held when controlling for date of challenge and date of egg collection (partial $r_2 = 0{\cdot}995$, $P = 0{\cdot}005$). The relationship between GnRH challenge response and yolk T is unlikely to have been directly caused by GnRH injection, as four of six eggs used in the analysis were collected from clutches that were laid weeks after the GnRH injection was administered (see Table 1) and the correlation remained strong when only these eggs were considered ($r_2 = 0{\cdot}98$, $P = 0{\cdot}02$).

During Egg Development, there was no significant correlation between yolk T and either initial ($r_4 = -0{\cdot}44$, $P = 0{\cdot}39$) or post-challenge plasma T ($r_4 = -0{\cdot}15$, $P = 0{\cdot}79$). This may indicate that yolk T more strongly covaries with HPG responsiveness rather than absolute levels of circulating plasma T.

Discussion

In this study, we investigated sources of variation among female dark-eyed juncos in the levels of plasma T observed during the reproductive season by analysing T produced following repeated GnRH challenges. Plasma T was measurable in females both before and after GnRH challenges during the entire breeding season. Overall, we found that females consistently showed an increase in plasma T in response to GnRH only in the week before laying their first egg of a clutch, which corresponds to a period of rapid yolk deposition in pre-ovulatory follicles. During other stages of reproduction, they failed to increase plasma T in response to GnRH, although in some cases this lack of response may be related to small sample size. This difference across reproductive stages appears to be due to changes at the level of the ovary, as a subset of individuals that did not respond to GnRH with an increase in plasma T did respond with an increase in LH in the early Spring (only time of LH sampling). Importantly, among a small number of females from which we collected eggs, the magnitude of the plasma T increase following GnRH challenge during egg development covaried positively with the level of T deposited in the egg yolk, but absolute levels of either initial or post-challenge plasma T did not covary with egg yolk T. This last finding is of interest in relation to gaining a greater understanding of where variation in egg yolk T may arise (see below). Further research, for example, increased sample sizes and finer temporal resolution is now needed to better understand how variation in yolk T occurs.

GnRH response during egg development

Plasma T reliably increased in response to GnRH only when females had the greatest number of hierarchical follicles, that is, the follicles that are the most active in producing steroids (reviewed in Johnson 2000). The increase in T in response to GnRH challenge that we observed may reflect stage-specific variation in the sensitivity of the pituitary to GnRH, the ovary to LH and/or a step in the conversion of T into oestradiol following ovarian stimulation. Such differences in hormonal responsiveness function in part to prepare for ovulation. Both androgen and oestradiol production are elevated during rapid yolk deposition and prior to the ovulation of eggs (Johnson 2000; Williams, Kitaysky & Vézina 2004). Progesterone and oestradiol in combination prime the hypothalamus and pituitary for the LH surge that initiates ovulation. Regardless of its relationship to the production of other hormones, however, our finding of a measurable GnRH-induced increase in plasma T during egg formation and its covariance with yolk T have interesting implications both for the developing egg and the adult, which we discuss below.

Covariation of plasma and egg yolk T

Our results suggest that variation among individuals in the magnitude of their response to GnRH and LH during egg development could account for variation in both the amount of T deposited into the yolk and also for changes in the amount of T in the female's plasma. If so, the T generated in response to GnRH and LH has the potential to influence attributes of the offspring via the yolk and the mother via the plasma. Such effects might be beneficial, detrimental or in conflict when viewed from the perspective of the mother or her offspring (reviewed in Gil 2003; Ketterson *et al.* 2005; Groothuis & von Engelhardt 2005).

Previous studies have shown that yolk T may covary with circulating T in the plasma (Schwabl 1996b), and that when plasma T is elevated experimentally, for example with implants, yolk T increases as well (Hackl *et al.* 2003; Clotfelter *et al.* 2004; Rutkowska *et al.* 2005, but see Navara *et al.* 2006). However, it is unclear whether T circulating naturally in the plasma during egg production represents 'excess' T coming from steroidogenic processes required for oogenesis or is related to the pre-ovulatory surge in levels of LH. Regardless of the specific mechanisms that drive correlations between plasma and yolk T, in juncos, the more strongly females

respond to GnRH challenges with increases in plasma T, the more strongly they may concentrate T in their eggs.

In a variety of species, females involved in more aggressive interactions, or living at greater densities, lay eggs with higher levels of yolk T (Schwabl 1997; Reed & Vleck 2001; Groothuis & Schwabl 2002; Whittingham & Schwabl 2002; Mazuc *et al.* 2003; Pilz & Smith 2004; Navara *et al.* 2006). If aggressive interactions stimulate the release of GnRH, the GnRH may in turn initiate the observed increases in yolk T. Similar social stimuli often lead to increases in circulating T in males (Wingfield 1985; Wingfield *et al.* 1990, 2001), and the levels of T released are predictable from the levels induced by challenge with GnRH (J.W. McGlothlin, unpublished). Evidence for such hormonal changes in females is mixed, however (Elekonich & Wingfield 2000; Hau *et al.* 2000; Langmore *et al.* 2002; Smith *et al.* 2005; Jawor *et al.* 2006a; Navara *et al.* 2006), and there is no direct evidence as yet that aggressive interactions cause the release of GnRH.

FEMALE PLASMA T AND ADULT BEHAVIOUR

Increases in plasma T in response to GnRH administration during egg production could have a variety of behavioural consequences in the adult female, all of which should be investigated further. For example, experimentally elevated T can increase aggression in female juncos (Zysling *et al.* 2006). Potentially, elevations in plasma T during egg production in female birds may enhance aggressive behaviour needed to secure nest sites, or vigilance needed to limit both intraspecific brood parasitism and extra-pair paternity of mates, thus maximizing limited resources for their progeny.

Increases in female plasma T during egg production may also affect mating behaviour. Females often show increased sexual behaviour, such as pre-copulatory displays, when given oestradiol, which is an aromatized metabolite of T (Leboucher, Kreutzer & Dittami 1994; Enstrom, Ketterson & Nolan 1997; Searcy & Capp 1997). Pharmacological blockade of the production of oestradiol from T in female canaries (*Serinus canaria*) (Leboucher *et al.* 1998) decreases solicitation displays, suggesting that elevated T could increase copulatory behaviour via its conversion to oestradiol. More copulation solicitations could increase the odds that each ovulated egg is fertilized and may promote higher levels of paternal assistance (see Kvarnemo 2005, Rios-Cardenas & Webster 2005; Albrecht, Kreisinger & Piálek 2006). Furthermore, increased T may affect a female's mate choice, which is most important when the female is fertile. Interestingly, female juncos show reduced choosiness between males when treated with exogenous T (McGlothlin *et al.* 2004).

REPEATABILITY OF RESPONSE

By sampling individuals repeatedly across the breeding season, we had hoped to investigate the repeatability of response in females to a GnRH challenge. In male Juncos, we have found that although plasma T response to GnRH challenge varies in intensity over the breeding season, the relative magnitude of an individual's response is repeatable (Jawor *et al.* 2006b). However, perhaps because females responded most strongly to GnRH only when producing eggs and our sample sizes for females known to be producing eggs was small, we were unable to detect consistent individual variation in the response. Repeatable variation among females may be more likely to be detected if females received repeated GnRH challenges during the egg development period across different clutches or breeding seasons. While it is too early to draw definitive conclusions, our data suggest that females could show consistency across clutches, because the covariation between GnRH-challenge response and yolk T remained strong and positive across successive clutches. However, if differences in GnRH response occur over the breeding season, maternal effects may differ across successive clutches and such variation across the season may be adaptive (Bowden, Ewert & Nelson 2000). For example, late season clutches, where offspring may not have much time to prepare for moult, migration or over-wintering, may benefit from the behavioural and developmental changes often associated with increased yolk T (Schwabl 1996a; Lipar & Ketterson 2000; Daisley *et al.* 2005; Eising, Müller & Groothuis 2006).

Conclusions

We conclude that in female juncos, GnRH-induced elevations in LH can increase T production from the ovary, but only when the ovary is primed to respond (e.g. when hierarchical follicles are present). These follicles appear to be responsive enough to produce T that is measurable in the circulation and in the yolks of developing eggs. Future studies should investigate the degree to which yolk T can increase in response to GnRH and LH increases and what effect this increase has on offspring development and behaviour. Also, whether female response to GnRH is repeatable over multiple clutches and how variation or consistency in T response influences offspring will need to be investigated. In addition, our results indicate that the relationship between T and adult female behaviour merits further exploration.

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