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Neural steroid sensitivity and aggression: comparing individuals of two songbird subspecies

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Abstract

Hormones coordinate the expression of complex phenotypes and thus may play important roles in evolutionary processes. When populations diverge in hormone-mediated phenotypes, differences may arise via changes in circulating hormones, sensitivity to hormones or both. Determining the relative importance of signal and sensitivity requires consideration of both inter- and intrapopulation variation in hormone levels, hormone sensitivity and phenotype, but such studies are rare, particularly among closely related taxa. We compared males of two subspecies of the dark-eyed junco (*Junco hyemalis*) for territorial aggression and associations among behaviour, circulating testosterone (T), and gene expression of androgen receptor (AR), aromatase (AROM) and oestrogen receptor α in three behaviourally relevant brain regions. Thus, we examined the degree to which evolution may shape behaviour via changes in plasma T as compared with key sex steroid binding/converting molecules. We found that the white-winged junco (*J. h. aikeni*) was more aggressive than the smaller, less ornamented Carolina junco (*J. h. carolinensis*). The subspecies did not differ in circulating testosterone, but did differ significantly in the abundance of AR and AROM mRNA in key areas of the brain. Within populations, both gene expression and circulating T co-varied significantly with individual differences in aggression. Notably, the differences identified between populations were opposite to those predicted by the patterns among individuals within populations. These findings suggest that hormone–phenotype relationships may evolve via multiple pathways, and that changes that have occurred over evolutionary time do not necessarily reflect standing physiological variation on which current evolutionary processes may act.

Introduction

Hormones mediate a variety of phenotypes that differ markedly among individuals and between species, including morphology, immune function, parental care and aggression (Adkins-Regan, 2005). Experimental manipulations of hormones in free-living animals suggest that hormones and the traits they mediate can have a profound effect on survival and reproduction in the wild (Salvador *et al.*, 1996; Reed *et al.*, 2006; Goutte *et al.*, 2010). Furthermore, natural variation in hormone profile has been shown to be associated with fitness and to be a target of selection (Breuner *et al.*, 2008; Bonier *et al.*, 2009; McGlothlin *et al.*, 2010). Collectively, these data suggest that hormones may play a central role in phenotypic evolution. However, the evolutionary mechanisms by which such hormone–phenotype relationships change as species diverge are poorly understood, but could have important implications for understanding how organisms respond and adapt to changing environments (Ball & Balthazart, 2008; Williams, 2008).

Artificial selection and hormone implant studies have provided evidence that natural selection on a hormone-mediated trait might result in the evolution of hormone

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titers themselves (Sinervo & Licht, 1991; Alonso-Alvarez, 2001; Marin & Satterlee, 2003; Evans *et al.*, 2006; Zera *et al.*, 2007; Williams, 2008; Mills *et al.*, 2009). Circulating hormones act on multiple targets throughout the body, and thus, evolutionary changes in hormone signal may affect many traits mediated by the same hormone, referred to as 'hormonal pleiotropy' (Ketterson & Nolan, 1999), the 'evolutionary constraint hypothesis' (Hau, 2007), or 'phenotypic integration' (McGlothlin & Ketterson, 2008; Ketterson *et al.*, 2009). On the other hand, the relationship between hormone titers and traits may change over evolutionary time, via an alteration in the degree to which traits are influenced by a given level of hormone, thus allowing selection to act on different hormone-mediated traits independently. This has been referred to as the 'evolutionary potential hypothesis' (Hau, 2007) or 'phenotypic independence,' (McGlothlin & Ketterson, 2008; Ketterson *et al.*, 2009; see also Hau & Wingfield, 2011). Endocrine mechanisms are complex, and, as a consequence, there are many factors that influence the degree to which hormones affect phenotype (e.g. receptor expression and affinity, carrier proteins, conversion enzymes and more [Ball & Balthazart, 2008; Wingfield, 2012]). Thus, in principle, there are many possible sources of variation in the endocrine system that could change over evolutionary time to alter hormone-mediated phenotypes, without changing circulating hormone levels themselves. Both phenotypic integration and independence likely play a role in the evolution of hormonal systems, but the nature of the interplay between the two remains unclear.

Research on the steroid hormone testosterone (T) has provided several useful examples for testing these hypotheses. Testosterone is generally elevated during the breeding season in vertebrates, and promotes traits associated with mating and resource defence, including aggression (Wingfield *et al.*, 1990; Ketterson & Nolan, 1992; Goymann *et al.*, 2007). Individual variation in circulating T has been shown to explain significant variation in aggressive behaviour (McGlothlin *et al.*, 2007), but there are also many exceptions where circulating T does not predict individual differences in aggression (reviewed in Adkins-Regan, 2005; Ball & Balthazart, 2008). It has been suggested that variability in neural sensitivity to T may be critical in explaining behavioural variation, particularly when circulating levels do not (Grunt, 1952; Adkins-Regan, 2005; Ball & Balthazart, 2008). Indeed, comparative studies of closely related species suggest that divergence may be accompanied by the uncoupling of certain phenotypic traits from T mediation, and thus may be one mechanism by which phenotypic independence is achieved (Hau, 2007; Lynn, 2008).

Researchers have often turned to neural expression of androgen receptor (AR), oestrogen receptors (ER) or the enzyme aromatase (AROM) when seeking to identify endocrine components that influence the degree to which a given T output yields a given phenotype. Testosterone can directly influence gene expression and thus phenotype directly, by binding to ARs in target tissues, or indirectly (after local conversion by AROM into 17β-estradiol [E_2]) via ERs. Gene knockouts and pharmacological manipulations strongly support linkages between these sex steroid binding/converting molecules and aggression (Ogawa *et al.*, 1997; Soma *et al.*, 1999b; Matsumoto *et al.*, 2003; Forlano *et al.*, 2006; Sperry *et al.*, 2010), and comparisons of phylogenetically or ecologically divergent groups have shown that differential expression of AR, ER or AROM in behavioural centres of the brain may account for phenotypic variation (Young *et al.*, 2006; Voigt & Goymann, 2007; Gonçalves *et al.*, 2010). Taken together, these studies suggest that the evolution of T-mediated trait expression can occur via changes in hormone sensitivity. Still, the ease with which these changes are achieved during divergence remains unclear, as these comparisons of neural sensitivity to sex steroids have typically focused on groups with substantial phylogenetic distance (Voigt & Goymann, 2007) or divergent life histories (Young *et al.*, 2006; Gonçalves *et al.*, 2010), but similar comparisons in closely related species are more rare.

We examined neuroendocrine mechanisms of aggression in two subspecies of the dark-eyed junco (*Junco hyemalis*), an abundant songbird that is thought to have diversified exceptionally rapidly following its radiation into North America in the last 10 000 years (Mila *et al.*, 2007). The Carolina subspecies (*J. h. carolinensis*) breeding in Virginia (VA) has been well characterized with respect to testosterone-mediated phenotype and fitness (e.g. Ketterson *et al.*, 1992; Reed *et al.*, 2006; McGlothlin *et al.*, 2010). Past work on this subspecies has shown that individual variation in both T and sensitivity to T play an important role in mediating aggressive behaviour (McGlothlin *et al.*, 2007; Rosvall *et al.*, 2012). Here, we contrast neuroendocrine mechanisms of aggression between free-living, breeding males from the VA subspecies to males of the less well-studied white-winged junco subspecies (*J. h. aikeni*) breeding in the Black Hills of South Dakota (SD). The SD subspecies is larger and more ornamented, but the two subspecies are otherwise similar in life history (Nolan *et al.*, 2002). Therefore, this subspecies comparison provides an excellent platform for examining how neuroendocrine mechanisms of aggression may change as populations diverge.

Specifically, we first asked whether these subspecies differed in levels of aggression directed towards a simulated territorial intruder. Next, we asked whether the subspecies differed in circulating T levels following this brief simulated territory intrusion (STI), and in the abundance of neural AR, AROM, or ERα mRNA. We focussed on gene expression for these receptors and enzyme because they approximate important components of local steroid sensitivity, although other

molecules also contribute to the immediate effects of steroids (Ball & Balthazart, 2008). Our study measures these estimates of steroid sensitivity in three behaviourally relevant areas of the brain that are known to be rich in abundance of sex steroid receptors (Metzdorf *et al.*, 1999; Zeigler & Marler, 2004; Goodson, 2005; Guerriero, 2009): the hypothalamus (HYPO), the right posterior telencephalon (PTR; including the song control nuclei) and the ventromedial telencephalon (VmT; including the avian medial amygdala or nucleus taeniae). Finally, we asked whether these populations differed in among-individual relationships between aggression and the endocrine parameters. By directly comparing individual and population variation in these endocrine parameters and their co-variation (or lack of co-variation) with behaviour, we examined the degree to which evolution can shape behaviour via changes in T, as well as changes in gene expression of key sex steroid binding/converting molecules. Generally, studies of neural steroid sensitivity have largely ignored the variation among individuals (but see Schlinger & Callard, 1989; Silverin *et al.*, 2004; Trainor *et al.*, 2006; Rosvall *et al.*, 2012), and we are not aware of any other studies that have examined the interindividual relationships between aggression and neural sensitivity to steroids across subspecies. This approach is needed to identify potential sources of functional individual variation and to provide insight into how selection may act (Arnold, 1983; Bennett, 1987; Williams, 2008).

Materials and methods

Field methods

Free-living, male dark-eyed juncos of the Carolina ($N = 17$) and white-winged ($N = 17$) subspecies were studied on their respective breeding grounds near the University of Virginia's Mountain Lake Biological Station in Virginia (37°22′N, 80°32′W), and in the Black Hills National Forest near Custer, South Dakota (43°46′ N 103°36′W). Territorial aggression was assayed with simulated territory intrusion (modified from McGlothlin *et al.*, 2007 for shorter length). All trials were conducted in the morning (0600–1200 local time) during the early- to mid-breeding season (VA: 5 May–5 June, SD: 14–22 May, 2010). The specific breeding stage of every male was generally not known (e.g. mated or unmated, mate fertile or incubating), but all males were confirmed to be in breeding condition post hoc by measuring gonads, and the two subspecies did not differ in any measure of gonad size (Christine Bergeon Burns, Kimberly Rosvall, Ellen Ketterson, unpublished data). Males from VA are the same males described in Rosvall *et al.* (2012), which investigated sex differences in endocrine mechanisms of aggression.

In the days preceding each aggression trial, individual males' territories were mapped by passive observation of focal male behaviour and by observations of behavioural responses to brief playback. On the day of each aggression trial, we placed a captive male lure (live decoy) in one cell of a four-cell potter trap, and placed the covered trap in the estimated centre of the focal male's territory. An iPod attached to a battery-powered speaker was placed directly next to the trap. One or two mist nets were set-up nearby and furled so as to not interfere with behaviour. We retreated 15 m, removed the cover from the lure and began song playback. One of 4 (VA) or 7 (SD) captive male juncos and one of 4 (each) unique junco song recordings were randomly selected for each aggression trial, at each respective study site. The recordings consisted of songs from the focal male's own subspecies, recorded in a previous year at an area distant from the focal male's territory. Each tape consisted of one long-range song type (Reichard *et al.*, 2011) repeated 9 times (90 s total), followed by a new song type repeated 9 times, and so on for a total duration of 6 min. Amplitude was standardized to be ~ 85–90 dB measured at 1 m.

One observer (KAR in VA, CMBB in SD) recorded the resident male's behaviour in response to this simulated territory intrusion for the duration of the trial. We quantified latency to arrive, number of flyovers (or dives) at the lure, number of songs sung, time spent near the cage and closest approach. Trials were abandoned if males did not appear within 2.5 min from start of trial, or if more than one male appeared. There was one trial per population where a second male appeared immediately after the trial, and we excluded behavioural measures from these two males, leaving $N = 16$ males per population with behavioural data.

At the end of each 6-minute behavioural trial, the three unoccupied cells of the potter trap were opened, the mist nets were rapidly unfurled, and the recording was switched to junco short-range song, a stimulus that elicits a very aggressive response (Reichard *et al.*, 2011). Birds were rapidly captured and killed by overdose of isoflurane followed by decapitation. Bodies were weighed, and trunk blood was collected in heparinized microhematocrit tubes (SD) or eppendorf tubes with 50 μL water-based heparin sodium salt solution added (VA) and stored on ice. Brains and gonads were dissected with RNAse-free sterile technique in the field, flash frozen in powdered dry ice, and stored at −80 °C.

Relative mRNA expression

At Indiana University, frozen brains were microdissected into 12 functional regions based on anatomical markers following Soma *et al.* (1999a). We focussed on three of these regions for RNA extraction: the VmT, which consists primarily of the nucleus taeniae (i.e. avian medial amygdala), the HYPO and the PTR, which consists of the song control regions. These regions were selected because they are known to be sensitive to

steroid hormone regulation, and have been associated with relevant social behaviours, including territorial song and aggressive approach (Vochteloo & Koolhaas, 1987; Metzdorf *et al.*, 1999; Zeigler & Marler, 2004; Goodson, 2005; Guerriero, 2009; Goodson *et al.*, 2012).

Total RNA was extracted from each individual brain region with the Trizol method (Invitrogen, Carlsbad, CA, USA). Quality and quantity of total RNA was measured with spectrophotometry, and 1 μg was treated with DNAse (Promega, Madison, WI, USA) and then reverse-transcribed with PCR into cDNA using oligo dT primers and Superscript III reverse transcriptase (Invitrogen). The cDNA was used as a template for quantitative real-time PCR (QPCR). Four RNA samples (2 VmT and 2 HYPO, all from SD) were low quality following extraction, and were omitted from analyses.

Quantitative real-time PCR was used to measure expression of AR, AROM and ERα in each brain region. We used the housekeeping gene Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) for normalization of the expression of each gene of interest. The subspecies did not differ in transcript abundance of this gene in any brain region ($F = 2.19$, $P = 0.15$). Gene-specific primers were based on zebra finch (*Taeniopygia guttata*) sequences (see Table S1 in the Supporting Information). By directly comparing zebra finch sequence to the recently sequenced junco transcriptome (Peterson *et al.*, 2012), we confirmed high sequence identity in all four genes (95%–98%).

Quantitative real-time PCR reactions (25 μL) were run in duplicate in a Stratagene MX3000p thermocycler (Agilent, Santa Clara, CA, USA) using Perfecta SYBR green low ROX, with 2.5 μL cDNA diluted 1 : 10 and primers at concentration of 0.3 μM. Thermocycling conditions were the same for all reactions, as follows: 10 min at 95 °C, 40 cycles of 95 °C for 30 s, 60 °C for 1 min and 70 °C for 30 s. A final dissociation phase of 95 °C for 1 min, 55 °C for 30 s and 95 °C for 1 min was run to confirm single-product specificity of each sample.

Standard curves were created from known cDNA dilutions. MxPro software (v.4.10; Agilent) was used to set thresholds for each reaction based on background fluorescence and to correct amplification data for imperfect reaction efficiencies (ranging from 93% to 116%). One cDNA sample derived from junco neural tissue was run on every QPCR plate, serving as a calibrator to which each individual sample was compared. Thus, we used the $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen, 2001), which reports relative abundance of transcript for each gene of interest relative to the calibrator, while controlling for the abundance of a reference gene (GAPDH).

Testosterone assay

Blood samples taken following brief STI were centrifuged on the day of collection, and the plasma fraction stored at −20 °C until T assays were conducted at Indiana University. An EIA kit (#ADI-901-065; Enzo Life Sciences International, Inc., Farmingdale, NY, USA) was used, as described previously (Clotfelter *et al.*, 2004). Briefly, 20 μL of plasma from each individual was diluted with water and spiked with a trace amount of ^{3}H-T (~ 2000 CPM). After two rounds of diethyl ether extraction, extracts were re-suspended in 50 μL ethanol and diluted with assay buffer to a volume of 350 μL. Hundred microlitre quantities were run in duplicate in EIA following manufacturer instructions. A four-parameter logistic curve-fitting program was used to determine T concentrations (Microplate Manager; Bio-Rad Laboratories, Inc., Hercules, CA, USA). One hundred microliters of extract was counted for ^{3}H decay in order to determine individual sample recovery of ^{3}H-T after extraction (average 90%). Testosterone concentrations were corrected to reflect incomplete recoveries, and where applicable we mathematically corrected for additional volume from water-based heparin solution. Three standards randomly distributed over each plate were used to calculate assay variation. The average intraassay coefficient of variation was 9.7%. Interassay coefficient of variation was 19.2%, and we applied a plate correction factor to correct for this interplate variability (Jawor *et al.*, 2007).

Statistical analyses

Statistical analyses were performed using SPSS Statistics 19 (IBM, Armonk, NY). We report mean ± SE unless otherwise noted, and all tests were two-tailed. In preliminary analyses, we tested for an effect of tape and decoy identity on song rate and flyover rate, and finding no effect in either population (ANOVA, all $F < 1.32$, all $P > 0.31$), we did not include these parameters as covariates in subsequent analyses. As subspecies differed behaviourally in latency to arrive after onset of an STI (SD: 0.95 ± 0.21 min, VA: 0.45 ± 0.15 min; Mann–Whitney U, $Z = -2.038$, $P = 0.0415$) and STIs were short, we standardized behavioural measures as a rate relative to the time each individual was confirmed to be present in the vicinity (e.g. within sight of the lure). As a consequence, exact statistical values reported for VA males differ slightly from Rosvall *et al.* (2012). We opted to focus our analyses on song rate and flyover rate to avoid any potential observer biases in distance measures, and because distance and arrival measures are more likely to be affected by known differences in habitat across the study sites (e.g. availability of perches or vegetation density, VA has a denser canopy and understory than SD). Testosterone data were ln-transformed and flyover rate was log transformed for normality. Relative transcript abundance for the gene of interest in the brain region of interest was log-base-2 transformed for normality where necessary.

Our experiment employed a short behavioural assay with the aim of relating unmanipulated gene transcription to behavioural responses. Thus, Pearson correla-

tions were employed to ensure that latency to sacrifice was unrelated to transcript abundance in any gene or brain area.

Testosterone, transcript abundance and behavioural measures were compared across subspecies with unpaired *t*-tests, and, where appropriate, accounting for unequal variance (identified by Levene's tests). Pearson correlations were used to examine interrelationships between transcript abundance for AR, ERα and AROM in each brain region of interest, and between measures of neural sensitivity and testosterone. The three measures of transcript abundance were significantly positively correlated in each brain region (see below). Thus, to avoid co-linearity of independent variables in subsequent general linear models (GLM), a single principal component was extracted separately for each brain region, representing steroid sensitivity. These principal components describe a large proportion of the variance among individuals in each brain region (67.3% in PTR, 70.5% in VmT and 85.4% in HYPO) and are loaded as in Table 1.

To explore relationships between behaviours and measures of sensitivity to T, we began with stepwise GLM. We analysed endocrine predictors of song rate and flyover rate in separate analyses because these two behavioural parameters were not significantly correlated (Pearson's $r = 0.10$, $P = 0.59$), and may serve different functions in an aggressive context (Wingfield *et al.*, 2006). We removed least significant predictors sequentially until highest overall model *F*-value was achieved. For models examining song rate as the dependent variable, subspecies were analysed together and we began with main effects of population and steroid sensitivity for each of the three brain regions (PCs), as well as interactions between each of the PCs and population. For models examining flyover rate as the dependent variable, we analysed subspecies separately because we identified a subspecies difference in flyover rate. In the initial flyover rate model, we began with main effects of steroid sensitivity for each brain region (PCs) as covariates.

We could not include T in the above step-wise model selection process without issues of co-linearity because T and sensitivity to steroids in the HYPO were correlated (see Table 1). Instead, we used a similar step-wise process to explore T as a predictor of aggression. As above, we analysed the subspecies separately for flyover rate because of differences in mean flyover rate.

Following initial GLM analyses, Pearson correlations were used to further explore relationships between significant endocrine parameters and behaviour. In particular, we dissected each PC that significantly predicted behaviour, looking for correlations between behaviour and mRNA abundance for AR, ERα and AROM separately.

Table 1 Loadings on the first principal component of neural steroid sensitivity. Principal component analysis was run separately for each brain region.

	PC1 loading for each brain region		
Transcript abundance	VmT	PTR	HYPO
AROM	0.818	0.813	0.922
ERα	0.917	0.873	0.934
AR	0.777	0.772	0.916
Eigenvalues	2.12	2.02	2.56

Results

Birds were captured an average of 4.7 ± 0.8 min following the end of the behavioural observation. Latency to capture did not differ between subspecies ($t = 0.40$, d.f. = 30, $P = 0.69$), nor was it related to transcript abundance for any gene in any of the brain areas (all $|r| < 0.31$, all $P > 0.1$), supporting our assumption that variation in gene expression within and between subspecies was unlikely to be a consequence of our behavioural assay.

Subspecies differences in endocrine parameters and behaviour

Subspecies differed in mRNA expression in behaviourally relevant brain areas. Males from VA had less AROM mRNA in HYPO ($t = 2.35$, d.f. = 29, $P = 0.026$), and more AR mRNA in VmT ($t = -3.85$, d.f. = 29, $P = 0.001$) than those from the SD subspecies (Fig. 1). The subspecies did not differ in any other measures of transcript abundance ($|t| < 1.03$, d.f. = 28–32, $P > 0.31$). Variance in circulating T levels was significantly greater in the SD subspecies (Levene's tests, $F = 6.79$, $P = 0.014$), but average T levels were not different between subspecies (VA: 2.69 ± 0.47 ng mL^{-1}; SD: 4.66 ± 1.11; $t = 1.34$, d.f. = 31, $P = 0.17$; Fig. 1).

Subspecies differed in aggressive behaviours directed at a simulated territorial intruder (Fig. 2): VA males performed fewer flyovers per minute than SD males (VA: 0.54 ± 0.12, SD: 1.02 ± 0.16; $t = 2.44$, d.f. = 30, $P = 0.021$). The two subspecies did not differ in the number of songs sung per minute (VA: 5.73 ± 0.71, SD: 5.29 ± 1.17; $t = -0.32$, d.f. = 30, $P = 0.76$), although SD males were significantly more variable in this behaviour (Levene's test, $F = 5.69$, $P = 0.024$).

Relationships among endocrine parameters

Transcript abundances for the three steroid-processing molecules were highly positively correlated within each brain region (AR vs. AROM HYPO: $r = 0.73$, $P < 0.001$, VmT: $r = 0.36$, $P = 0.047$, PTR: $r = 0.43$, $P = 0.006$; AR vs. ERα HYPO: $r = 0.79$, $P < 0.001$, VmT: $r = 0.61$, $P < 0.001$, PTR: $r = 0.53$, $P = 0.002$;

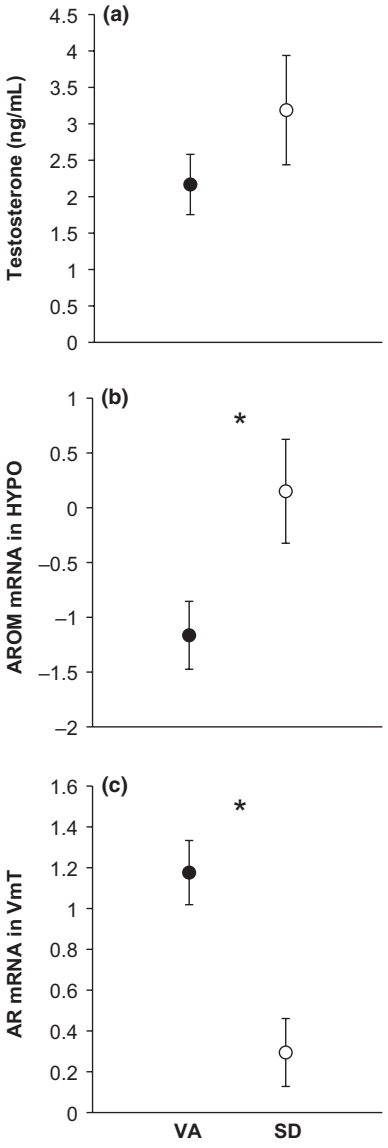

Fig. 1 (a) Populations did not differ in average T levels following brief STI, but (b) South Dakota males (open circles) had significantly more AROM mRNA in the ventromedial telencephalon and (c) Virginia males (filled circles) had significantly more AR mRNA in the hypothalamus. Testosterone levels represent back-transformed means. Measures of transcript abundance are $\log_2$-fold change relative to arbitrary calibrator (unitless). Figures show means ± 1 standard error. Significant differences denoted by asterisk (*).

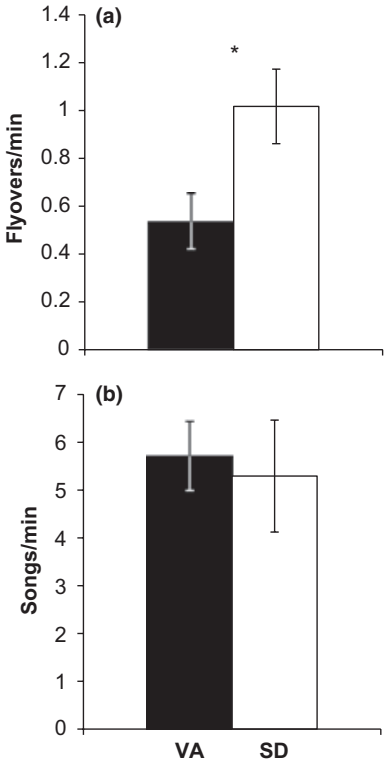

Fig. 2 (a) South Dakota males (open bars) performed significantly more flyovers at the live decoy than Virginia males (filled bars), but (b) the subspecies did not differ the number of songs sung. Song rate and flyover rate were both adjusted for the total time that the male was present during the 6-minute stimulated territorial intrusion. Figures show means ± 1 standard error. Significant differences denoted by asterisk (*).

AROM vs. ERα HYPO: $r = 0.80$, $P < 0.001$, VmT: $r = 0.67$, $P < 0.001$, PTR: $r = 0.59$, $P < 0.001$). Plasma concentration of T was significantly negatively correlated with gene expression in HYPO (AR: $r = -0.43$, $P = 0.017$, ERα: $r = -0.35$, $P = 0.059$, AROM: $r = -0.44$, $P = 0.014$, PC1: $r = -0.44$, $P = 0.016$; Fig. 3), but not in VmT or PTR (all $|r| < 0.31$, all $P > 0.09$). The relationships were qualitatively similar when subspecies were analysed separately (See Table S2).

Patterns of covariation between endocrine parameters and behaviour

General linear models with song rate found no significant main effects of the first principal components of steroid sensitivity in each brain region, but did reveal a significant interaction between population and PC1 HYPO (Table 2). Subsequent Pearson correlations on the two subspecies separately revealed a significant negative relationship between PC1 HYPO and song rate among males in VA ($r = -0.611$, $P = 0.016$), but not in SD ($r = -0.08$, $P = 0.78$; Fig. 4). Further analysis of each gene separately reveals that this relationship with song rate appears to be consistent across each of the steroid-processing molecules in VA (HYPO: AROM: $r = -0.64$, $P = 0.008$; ERα: $r = -0.50$, $P = 0.056$; AR: $r = -0.54$, $P = 0.030$) but no such relationship exists in the SD subspecies (all $|r| < 0.14$, all $P > 0.63$). Interestingly,

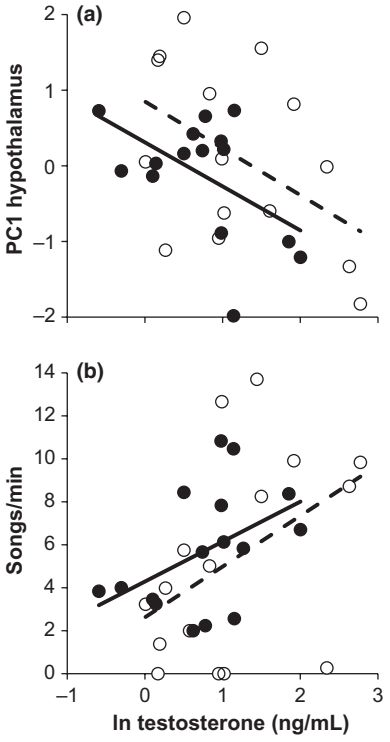

Fig. 3 In both populations, (a) sensitivity to sex steroids in the hypothalamus, measured as the first principal component of transcript abundance for AR, ERα and AROM, was negatively related to circulating testosterone levels, and (b) song rate was positively related to circulating T. Testosterone was ln-transformed for normality, and song rate was corrected for time present during the STI. Each dot represents an individual male. Virginia (filled circles, filled line) and South Dakota (open circles, dashed line) males were plotted separately.

previous analyses of VA males also identified positive correlations between number of songs sung and transcript abundance of both ERα and AROM, but not AR, in PTR (Rosvall *et al.*, 2012); however, this relationship was not statistically significant here, likely due to the use of principal components.

With respect to flyover rate, GLM pointed to PC1 HYPO, but not VmT or PTR, as a significant predictor in the SD subspecies (Table 2, Fig. 4). Subsequent Pearson correlations confirmed the significant negative relationship with flyover rate (PC1: $r = -0.64$, $P = 0.013$) and suggested that the relationship is consistent across each gene of interest (HYPO: $r = -0.64$, $P = 0.015$; ERα: $r = -0.52$, $P = 0.058$; AR: $r = -0.69$, $P = 0.006$). In VA, the best GLM indicated a marginally significant main effect of PC1 VmT (Table 2). Subsequent Pearson correlations revealed a positive correlation between flyover rate and transcript abundance for ERα in VmT ($r = 0.52$, $P = 0.047$), but relationships were not significant for AR ($r = 0.46$, $P = 0.074$) or AROM ($r = 0.31$, $P = 0.239$).

Table 2 General linear models exploring the effects of neural sensitivity to steroids on song and flyover rates in *Junco hyemalis* males. Models with best fit are presented and significant *P*-values are indicated in bold.

Factor	d.f.	*F*	Significance
Song rate			
Overall	3, 22	2.16	0.122
PC1 HYPO	1, 22	0.78	0.386
Population * PC1 HYPO	1, 22	5.19	**0.033**
Population	1, 22	1.08	0.310
Flyover rate			
South Dakota			
PC1 HYPO	1, 12	8.44	**0.013**
Virginia			
PC1 VmT	1, 13	4.38	0.057

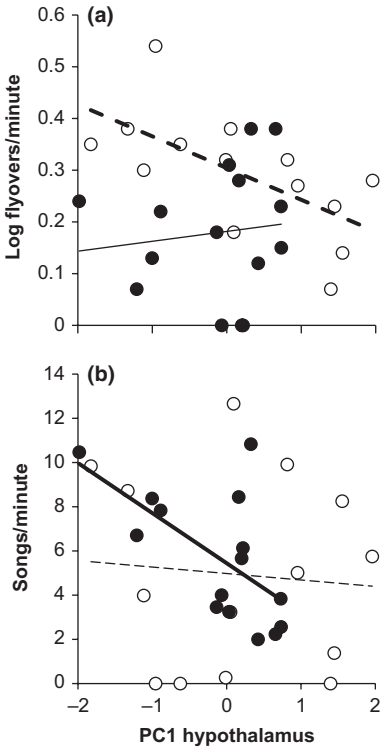

Fig. 4 Hypothalamic sensitivity to sex steroids, measured as the first principal component of AR, ERα and AROM mRNA abundance, was negatively correlated with (a) song rate in Virginia and (b) flyover rate in South Dakota. Flyovers were log-transformed for normality. Behavioural rates were corrected for time present during the STI, and each dot represents one individual male. Virginia (filled circles, filled line) and South Dakota (open circles, dashed line) males were plotted separately. Thick lines are statistically significant, whereas thin lines are not.

Testosterone was identified as a significant predictor of song rate using GLM, with no significant main effect of, or interaction with, population (Table 3, Fig. 3).

Table 3 General linear models exploring the effects of testosterone on song and flyover rates in *J. hyemalis* males. Models with best fit are presented and significant *P*-values are indicated in bold.

Factor	d.f.	*F*	Significance
Song rate			
Testosterone	1, 30	6.55	**0.016**
Flyover rate			
South Dakota			
Testosterone	1, 14	1.48	0.244
Virginia			
Testosterone	1, 14	0.40	0.538

Subsequent Pearson correlations confirm that this positive relationship holds up in each subspecies (VA: $r = 0.45$, $P = 0.077$; SD: $r = 0.45$, $P = 0.080$), although statistical significance was achieved only when they were combined ($r = 0.42$, $P = 0.016$; Fig. 3). Testosterone did not significantly predict flyover rate in either subspecies (Table 3).

Discussion

In comparing two subspecies of junco, we found robust differences in behaviour and in transcript abundance for sex steroid binding/converting molecules in the brain. Within populations, variation in both circulating T and in some measures of neural sex steroid sensitivity were correlated with individual differences in aggression, and some of these patterns of co-variation were shared between populations. Collectively, these similarities and differences support the view that the relationships between behaviour and expression of steroid-processing genes can shift as populations diverge, even between very recently diverged subspecies. This study is among the very few direct comparisons of intrapopulation mechanisms of aggression across ecologically similar but phenotypically divergent populations. As a consequence, the results provide important insights into potential mechanisms underlying hormone-mediated trait divergence.

Subspecies differences in endocrine parameters and behaviour

The larger and more ornamented white-winged junco of SD (Nolan *et al.*, 2002) was more aggressive than the Carolina junco of VA, as measured by the rate of flyovers during simulated territorial intrusions. Contrary to expectation of T-mediated phenotypic integration, we did not observe higher levels of plasma T in the more aggressive subspecies. As blood was sampled following a behavioural assay, we cannot assume that these T levels reflect baseline values, although they also are not likely to reflect maximal T output given the short (6 min) duration of STI. It remains to be seen whether $T_{potential}$ (i.e. response to GnRH challenge, per Goymann *et al.*, 2007) does or does not vary between these subspecies. The two populations did differ in the abundance of AR and AROM mRNA in brain areas known to be associated with aggression. Males in the VA subspecies had less AROM mRNA in HYPO and more AR mRNA in VmT than males in the SD subspecies. One possible interpretation of these data is that the oestrogenic pathways may be more important in the SD subspecies; however, the subspecies did not differ in the abundance of ERα mRNA in any neural tissues examined.

Although we cannot yet say whether these subspecies differences in mRNA translate to differences in protein, our observations are consistent with the view that components of endocrine-mediated behavioural systems can diverge independently of changes in circulating levels of hormones. Notably, our findings differ in a number of ways from what we might have predicted. For example, compared with their SD counterparts, VA males display similar T levels despite what might represent less feedback inhibition in the HYPO (e.g. less AROM mRNA), and lower average aggression despite what appears to be increased sensitivity in a brain area associated with aggression (e.g. more AR mRNA in the VmT). This differs from earlier work that suggested more steroid receptors ought to increase neural response to circulating T, and therefore be positively associated with aggression (Canoine *et al.*, 2007; Voigt & Goymann, 2007). Further experiments are needed at finer spatial scales within the HYPO and VmT to reveal whether receptor densities within various subnuclei may drive the patterns we found.

Relationships among endocrine parameters

Few studies have addressed individual variation in abundance of steroid-processing molecules (Ball & Balthazart, 2008). Here, we found that T levels among males were largely uncorrelated with AR, AROM and ERα transcript abundance in the brain, with the exception that T was negatively correlated with AROM mRNA in HYPO in both populations (discussed below). Interestingly, AR, AROM and ERα mRNA generally showed strong positive correlations with one another within a tissue. These findings suggest some potential for signal and sensitivity to vary independently, although they also reveal a striking integration of multiple measures of sex steroid sensitivity in the brain.

Patterns of covariation between endocrine parameters and behaviour

Both populations showed a negative relationship between hypothalamic sensitivity to steroids and aggressive behaviour, although this relationship was detected for song rate in VA and flyover rate in SD.

Our results add to the small but growing number of studies linking individual variation in aggression with individual differences in gene expression for the molecules that bind/convert sex steroids in the brain (Schlinger & Callard, 1989; Silverin *et al.*, 2004; Trainor *et al.*, 2006; Goodson *et al.*, 2012; Rosvall *et al.*, 2012). Several nuclei within the HYPO have been linked with aggression (ventromedial hypothalamus preoptic area, etc.); however, contrary to our findings among individuals, reduced aggression is typically associated with experimentally inactivated AR, ERα or AROM (Ogawa *et al.*, 1997; Soma *et al.*, 1999b; Matsumoto *et al.*, 2003; Sperry *et al.*, 2010) or lower abundance of these molecules in comparisons made among species (Goodson *et al.*, 2012; Voigt & Goymann, 2007; but see Gonçalves *et al.*, 2010). Our unexpected findings may be a reflection of differences across multiple nuclei because not every AR-expressing cell in the HYPO is the same, and not every one relates to aggressive or social behaviour. Notably, the HYPO contains the integrating centre for activation of the HPG axis and is known to be a target of negative feedback of steroids (Bagamasbad & Denver, 2011). We found a significant negative correlation between PC1 in the HYPO and circulating T. Thus, it is possible that the negative relationships we detected between hypothalamic AROM expression and aggression are indirect, and may actually reflect the positive relationship we detected between T and aggression. Our observations nevertheless suggest a good deal of similarity in brain-behaviour co-variation across divergent subspecies.

In the tissues containing the medial amygdala, we found a significant positive relationship among VA males between flyover rate and neural sensitivity to steroids, most strongly ERα. This is consistent with previous studies that have suggested that greater steroid sensitivity in this region is associated with social behaviour (Canoine *et al.*, 2007; Voigt & Goymann, 2007), likely through its influence on motivational state (Cheng *et al.*, 1999). It is not yet clear why these relationships were only identified in the VA subspecies. One interpretation is that the subspecies with less variability in T has co-opted additional mechanisms of aggression, such that differences in neural sensitivity to sex steroids are more important to the expression of aggression in VA than in SD. In any case, our data demonstrate that intrapopulation patterns of co-variation between neural sensitivity to steroids and aggressive behaviour exist even in the absence of co-variation with T.

Phenotypic integration and independence in endocrine mechanisms

Our comparison of these two subspecies of junco may provide some resolution to the relative influence of phenotypic integration and independence in the divergence of species. Some components of our comparison of mean phenotype across subspecies are consistent with phenotypic integration: the larger and more ornamented (Nolan *et al.*, 2002) SD subspecies was also significantly more aggressive, as measured by flyover rate, than their VA counterparts. In addition, we found a conserved pattern of individual co-variation between aggression (singing rate) and T across the two subspecies. These patterns are consistent with previous accounts from this and other species of a relationship between plasma androgens and behaviours that are relevant during social instability (Wingfield *et al.*, 2001; Hirschenhauser & Oliveira, 2006; Goymann *et al.*, 2007), and they suggest that individual differences in circulating T may regulate song output in similar ways in the two subspecies.

On the other hand, we found that circulating levels of T were not significantly different in SD and VA when measured at comparable stages of the breeding season, despite subspecies differences in aggressive approach. Regulation of steroid hormone binding/conversion at the target tissue has been suggested as one mechanism by which hormone-mediated traits may evolve independently (Ketterson *et al.*, 2009). Our results provide support for this hypothesis: subspecies differed in gene expression for AR and AROM in some areas of the brain, and we identified individual co-variation between behaviour and transcript abundance for sex steroid binding/converting molecules. Importantly, T did not relate to measures of target tissue sensitivity among individuals in the song control nuclei (PTR) or medial amygdala (VmT). These results point to great potential for independent evolution of endocrine components under selection.

Comparing patterns at individual and population levels of analysis: implications for understanding phenotypic divergence

Identification of individual co-variation between traits and the mechanisms that give rise to them is critical to understanding how hormone-mediated traits might evolve, as it identifies sources of variation upon which selection may act (Arnold, 1983; Bennett, 1987; Williams, 2008), and changes is gene expression are thought to be a major driver of evolutionary change (Whitehead & Crawford, 2006). Thus, it is interesting to note that the patterns we identified *between populations* – both with respect to signal and sensitivity relating to phenotype – did not always mirror the differences we observed *among individuals* within one population, and, in fact, they were just the opposite. Specifically, among individuals, *more* aggressive males showed less steroid sensitivity in the HYPO, and greater sensitivity in VmT. Yet, when comparing subspecies, the *less* aggressive VA subspecies showed less sensitivity in the HYPO, and greater sensitivity in VmT. These different inter- and intrapopulation patterns may be a

reflection of past versus current evolutionary processes, including potential differences in selective pressures over space and time, and/or stochastic evolutionary processes. Phylogenetic analyses suggest that founder effects may have contributed to the differentiation of the white-winged subspecies during the rapid post-glacial radiation of juncos (Mila *et al.*, 2007). Although our study was limited to two subspecies of a rapidly diverging lineage, inclusion of additional populations and extension to other taxa will be necessary to test the generality of these findings.

Clearly, there is much to be learned about the relative lability and interconnectedness of endocrine mechanisms as populations diverge. Our data suggest that the components of the endocrine system that *are currently* variable and could be shaped by evolutionary processes within each subspecies may not necessarily reflect those components that *were previously* variable or predictive of behaviour during divergence. Population and species comparisons are not new to the field of behavioural neuroendocrinology (Young *et al.*, 2006; Gonçalves *et al.*, 2010), but past research has focused largely on group means, without also contrasting interindividual patterns of co-variation between neuroendocrine and behavioural parameters. Our complex findings emphasize that future work should examine individual variation when comparing groups, instead of assuming that intergroup patterns will mirror interindividual patterns within a group, as these multiple perspectives allow for richer insights into the mechanisms of divergence.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Table S1 Primer sequences, amplicon size, and GenBank accession numbers for the zebra finch (*T. guttata*) source files.

Table S2 Pearson's correlations among endocrine components, analyzed separately for each population.

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