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Examining sources of variation in HPG axis function among individuals and populations of the dark-eyed junco

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ABSTRACT

Gonadal steroids are important mediators of traits relevant to fitness, and thus may be targets of selection. However, more knowledge is needed about sources of variation along the endocrine axes that may contribute to functional variation in steroid levels. In a controlled captive environment, we studied males of two closely related subspecies of the dark-eyed junco (*Junco hyemalis*) that differ in testosterone-related phenotype, asking whether they also differ in testosterone (T), and assessing the contribution of the sequential links of the hypothalamic–pituitary–gonadal axis. When males of both subspecies were challenged with gonadotropin-releasing hormone (GnRH), they were similar in circulating luteinizing hormone (LH) and T responses. When challenged with exogenous LH, they again produced levels of T similar to one another, and to the levels produced in response to GnRH. However, the smaller, less ornamented, and less aggressive subspecies had greater abundance of mRNA for LH receptor in the testes and for androgen receptor in the rostral hypothalamus, suggesting potential differences in regulatory feedback. We suggest that circulating hormone levels may be less prone to evolutionary change than the responsiveness of individual hormone targets. Among individuals, T titers were highly repeatable whether males were challenged with GnRH or with LH, but LH produced in response to GnRH did not covary with T produced in response to LH. Testis mass, but not LH receptor transcript abundance, predicted individual variation in T responses. These data implicate the gonad, but not the pituitary, as an important source of individual variation in T production.

Introduction

The gonadal steroid testosterone (T) has profound effects on multiple morphological, physiological and behavioral traits throughout the brain and periphery, promoting traits such as sperm production, aggression, sexual behaviors and ornaments, sometimes at the expense of immunity and parental care (Folstad and Karter, 1992; Ketterson et al., 1992; Wingfield et al., 2001). Accordingly, gonad size and circulating concentrations of T often fluctuate seasonally along with these phenotypes. In temperate-breeding songbirds, circulating T is typically higher during territory establishment and mating, and lower during nesting and non-breeding (Goymann et al., 2007; Moore et al., 2002; Wingfield et al., 1990). T levels are also dynamic on finer time scales throughout the breeding season, associated with enhanced aggression or sexual behavior during male–male and male–female social interactions (Ball and Balthazart, 2004; Goymann et al., 2007; Wingfield et al., 1990). Individuals and species vary in T and related phenotype, and it has been demonstrated that individual variation in T has important consequences for fitness (e.g., McGlothlin et al., 2010; Veiga and Polo, 2008). Despite the potential role for hormonal systems in phenotypic evolution, relatively little research has addressed the mechanistic sources of variation in T titers and T signaling pathways upon which selection may act (Ball and Balthazart, 2008).

The neuroendocrine system responsible for T regulation is complex, and it is important to consider the multiple potential sources of variation in this system in order to understand the mechanistic underpinnings of T-mediated trait evolution (Hau and Wingfield, 2011). Most of the phenotypic changes associated with reproduction in birds are consequences of the regulation of the hypothalamic–pituitary–gonadal (HPG) axis. Environmental cues are relayed to the hypothalamus, eliciting an increase in the release of gonadotropin-releasing hormone (GnRH). GnRH triggers secretion of gonadotropins such as luteinizing hormone (LH) from the anterior pituitary, which in turn acts at the gonad via the G-protein coupled luteinizing hormone receptor (LHR), stimulating the synthesis and release of steroids including testosterone (T) from the gonads. Like LH, T must be transduced by receptors to have its effects (Hadley and Levine, 2007), and this process may be influenced by many variables including carrier proteins, conversion enzymes and co-factors, as well as receptor expression and affinity. T typically acts via intracellular receptors located at many

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targets throughout the brain and periphery, either by activating androgen receptors (ARs, binding directly or after conversion to 5α-dihydrotestosterone), or by being converted by the enzyme aromatase to 17β-estradiol (E_2), which binds to estrogen receptors (ERs). When bound, these receptors function as transcription factors to regulate gene expression, affecting various physiological, morphological or behavioral processes (Ball and Balthazart, 2008; Wingfield, 2012).

Whereas the HPG axis is often visualized as acting top down, it is in fact a dynamic self-regulating system, sensitive to feedback and modulation at multiple levels. Early studies of the mechanisms underlying T-mediated trait expression focused on the gonad, while more recent research has emphasized mechanisms of steroid action in the brain (Adkins-Regan, 2005; Ball and Balthazart, 2008; Rosvall et al., 2012; Soma, 2006), including the many stimulatory and inhibitory afferents in the brain that act to integrate environmental responses and relay them to the level of the GnRH neuron, leading to downstream HPG activation (e.g., Bentley et al., 2009). Despite considerable advances in understanding the complexity of these constituent parts, little work has integrated multiple levels of the HPG axis simultaneously in an evolutionary framework. It has been theorized that the brain may be best suited to respond to selection on T-mediated traits via adjustments in the way environmental stimuli elicit GnRH release, while the responses of the downstream endocrine portions of the HPG axis may be less likely to be altered during evolution (Adkins-Regan, 2008). However, evidence that directly points to the sources of individual or population variation along the HPG axis is lacking.

GnRH challenges, in which individuals are administered a standardized dose of exogenous GnRH, essentially bypass the brain as a source of variation to measure downstream capacity to secrete androgens (see Goymann et al., 2007). Recent work in *Zonotrichia* has identified population differences in the ability to modulate T in response to social stimuli, despite similar propensity to elevate T following GnRH challenge (e.g., Addis et al., 2011), suggesting that divergence in HPG activity may occur at the level of the brain. However, studies comparing individual responses to GnRH injections have demonstrated functional variation in T that likely originates downstream of the brain: Variation in T response to GnRH challenge is repeatable among individuals (Jawor et al., 2006), maps onto phenotypic traits (McGlothlin et al., 2007, 2008), and is under selection (McGlothlin et al., 2010). This suggests that variation in the pituitary–gonadal axis downstream of GnRH may contribute to differences among individuals and be an important target of selection.

In order to better understand the functional mechanisms by which variation in the HPG axis may be translated to phenotype, multiple levels of the endocrine system must be examined collectively as potential sources of variation in T. Any one or many of these components could vary among individuals, sexes, populations, or species, leading to variation in T and fitness. Importantly, it is unknown whether selection acts on different endocrine components as one integrated unit or separately as independently varying targets, which could have important implications for predicting responses to selection (Adkins-Regan, 2008; Hau, 2007; Hau and Wingfield, 2011; Ketterson et al., 2009). Comparing sources of variation in multiple endocrine components along multiple levels of the HPG axis across groups is a promising approach that has not yet been adequately explored.

Exceptions include work on alternative phenotypes. Research on white-throated sparrows (*Zonotrichia albicollis*), which have a chromosomal inversion resulting in a behavioral polymorphism, find morph differences in T but conflicting evidence as to whether differences are also reflected in upstream LH signal from the pituitary (Lake et al., 2008; Spinney et al., 2006). Research on the African cichlid *Astatotilapia burtoni*, which displays environment-induced dominant and subordinate phenotypes, has similarly looked at sources of variation along multiple levels of the HPG axis, and has suggested depression of the entire HPG axis in subordinates as compared to dominant individuals (Maruska and Fernald, 2010; Maruska et al., 2010). While these comparisons of genetic and environmentally induced morphs provide important insights into the mechanisms underlying phenotypic diversity, the relative lability and interconnectedness of endocrine components under selection remain unknown. From an evolutionary perspective, it is important to examine sources of variation in the HPG axis that may be shaped by selection. Artificial selection on white-footed mice (*Peromyscus leucopus*) for high and low reproductive suppression in winter has found genetic variation in the brain as well as in downstream pituitary LH release (Heideman and Pittman, 2009; Heideman et al., 2010). Recent work by Caro et al. (2006, 2009) has begun to identify mechanisms underlying population differences in the timing of breeding in Corsican blue tits (*Cyanistes caeruleus*). Still, studies like these are few, and those comparing individuals of naturally divergent populations are lacking.

Here, we contrast activity of the HPG axis of two subspecies of a songbird, the dark-eyed junco (*Junco hyemalis*). The post-glacial diversification of the junco is thought to be exceptionally extensive given the relatively short timescale and may represent incipient speciation (Mila et al., 2007). Thus this species provides an excellent system for the comparative study of endocrine mechanisms. Using captive male birds in breeding condition, we examined the degree to which individual and subspecies differences in T released in response to GnRH originate at the level of the pituitary, at the level of the gonad, or both. Using traditional measures of circulating hormones and modern molecular methods to assess measures of hormone sensitivity, we examined circulating LH and T responses to HPG axis stimulation, transcript abundance for LHR in the testes and testicular development, as well as both AR and AROM transcript abundance in the rostral hypothalamus, a potentially important site of negative feedback regulating T release. We explored the degree of interconnectedness (integration) among these endocrine parameters, as well as how they diverge between subspecies.

We compared the Carolina subspecies of junco (*Junco hyemalis carolinensis*) from a population that breeds around Mountain Lake Biological Station near Pembroke, Virginia, USA (37°22′N, 80°32′W), and the white-winged junco (*Junco hyemalis aikeni*) from a population that breeds in the Black Hills National Forest near Custer, South Dakota, USA (43°46′N 103°36′W). The initiation of breeding differs by approximately one month in these subspecies (first eggs typically appear during mid- to late April in Carolina juncos, latter half of May in white-winged juncos), while the timing of the end of breeding is similar with first signs of molt appearing in both species in July (Nolan et al., 2002; Bergeon Burns and Ketterson, unpublished data).

Studies conducted on Carolina juncos have provided a wealth of information about T-mediated phenotypic traits and trade-offs (McGlothlin et al., 2010; Reed et al., 2006). Juncos vary in the degree to which they elevate T in response to a standardized injection of GnRH, and maximum T levels produced after GnRH challenges decline across the breeding season and are repeatable among individuals (Jawor et al., 2006). Further, T responses co-vary with phenotypic characters, such as ornamentation and body size, and predict reproductive success (McGlothlin et al., 2007, 2010), presenting an ideal opportunity for examining the mechanisms underlying known individual variation in these fitness-relevant traits. The white-winged subspecies was chosen for comparison with the Carolina juncos because white-winged males have the largest body size and highest levels of ornamentation as compared to any other population of dark-eyed junco (Nolan et al., 2002), raising questions about the role for androgens in the population divergence.

We tested the hypothesis that T levels may contribute to population differences in body size and ornamentation in juncos, and we predicted greater T response to GnRH challenge in the larger and more ornamented white-winged junco subspecies. We hypothesized that selection acts on the HPG axis as one integrated unit, such that potential differences between these divergent populations in T production could be attributed to differences across each level of the HPG axis examined.