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Divergence along the gonadal steroidogenic pathway: Implications for hormone-mediated phenotypic evolution

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ABSTRACT

Across a range of taxa, hormones regulate suites of traits that influence survival and reproductive success; however, the mechanisms by which hormone-mediated traits evolve are still unclear. We hypothesized that phenotypic divergence might follow from differential regulation of genes encoding key steps in hormone biosynthesis and thus the rate of hormone production. We tested this hypothesis in relation to the steroid hormone testosterone by comparing two subspecies of junco (*Junco hyemalis*) in the wild and in captivity. These subspecies have diverged over the last 10–15 k years in multiple testosterone-mediated traits, including aggression, ornamentation, and body size. We show that variation in gonadal gene expression along the steroid biosynthetic pathway predicts phenotypic divergence within and among subspecies, and that the more androgenized subspecies exhibits a more prolonged time-course of elevated testosterone following exogenous stimulation. Our results point to specific genes that fulfill key conditions for phenotypic evolution because they vary functionally in their expression among individuals and between populations, and they map onto population variation in phenotype in a common garden. Our findings therefore build an important bridge between hormones, genes, and phenotypic evolution.

Steroid hormones are chemical messengers that link environmental stimuli with the expression of a variety of traits across a range of taxa, including growth and immunity in plants (Oklestkova et al., 2015), color and size in insects (Oostra et al., 2011), and many social and sexual behaviors in vertebrates (Adkins-Regan, 2005; O'Connell and Hofmann, 2012). Because these effects are often mediated via hormonal activation of gene activity, hormones can influence how genotype is translated into phenotype, generating the potential for hormones to be proximate mediators of phenotypic evolution (Ketterson and Nolan, 1999; Zera et al., 2007). In vertebrates, the sex steroid testosterone (T) has been experimentally linked with survival, reproductive success, and many components of phenotype (Ketterson et al., 1992; Reed et al., 2006; Sinervo et al., 2000). Artificial selection on circulating T levels can influence hormone-mediated phenotypes and fitness (Mills et al., 2012; Robison et al., 1994; Walker et al., 2004), and interspecific differences in T profiles correspond to divergence in other traits as well (Dijkstra et al., 2012; Goymann et al., 2007). Thus, changes in circulating T have the potential to bring about phenotypic evolution (Hau, 2007; Ketterson et al., 2009; Wingfield, 2012); however, the underlying mechanisms by which these changes occur remain unclear.

There are several reasons for this lack of clarity. First, like many hormones, T is not a direct gene product, but rather the product of a multi-enzyme pathway and complex endocrine cascade. The hypothalamo-pituitary-gonadal (HPG) axis is activated when an environmental cue, such as day length or a conspecific, stimulates the hypothalamus to secret gonadotropin-releasing hormone (GnRH). GnRH acts on the pituitary to secrete gonadotropins, including luteinizing hormone (LH), which stimulates the gonad to produce T, which is derived from cholesterol via several intermediates. Further complexity arises via feedback along the HPG axis, variation in hormone receptor densities in target tissues, and other factors that exhibit plasticity in response to the environment (Ball and Balthazart, 2008). This plasticity poses a challenge for evolutionary biologists seeking to understand how hormone-mediated traits evolve because plasticity can obscure otherwise consistent individual variation that represents the raw material of evolutionary change (Whitehead and Crawford, 2006).

Here, we sought to identify mechanistic sources of variation in T production, within and among populations, concentrating on gene expression related to the biosynthesis of T in the gonad (Fig. 1). Empirical and theoretical research on other biosynthetic pathways suggests that genes whose products are located early in a pathway or those catalyzing multiple, branching steps ought to be major regulators of flux through these pathways (Rausher, 2013; Wright and Rausher, 2010). As a consequence, we focused on steroidogenic acute regulatory protein (StAR),

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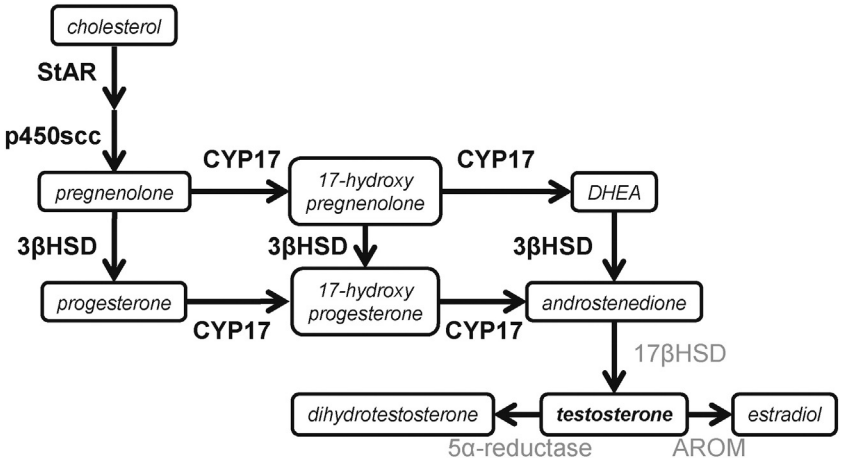

Fig. 1. A simplified overview of testosterone synthesis. Genes of interest are highlighted in bold. StAR = steroidogenic acute regulatory protein. P450scc = Cytochrome P450 side-chain cleavage; CYP17 = Cytochrome P450 17α-hydroxylase; 3βHSD = 3β-hydroxysteroid dehydrogenase/isomerase; 17βHSD = 17β-hydroxysteroid dehydrogenase. DHEA = dehydroepiandrosterone. AROM = aromatase.

cytochrome p450 side-chain cleavage (p450scc), 3-β-hydroxysteroid-dehydrogenase (3βHSD), and cytochrome p450 17α-hydroxylase (CYP17), due to their position in the pathway to produce T. In addition, all have been linked with variation in T secretion and T-mediated phenotypes in other contexts, e.g. during ascent to social dominance in a cichlid fish (Huffman et al., 2012) or in association with hormonal disorders in humans (LaVoie and King, 2009; Payne and Youngblood, 1995). However, we are not aware of any study that has identified these candidate genes as potential sources of within-sex individual differences in T. We hypothesized that differences in the expression of these genes would underlie individual and population variation in the regulation of T and the expression of T-mediated traits.

The subject of this research is the dark-eyed junco (*Junco hyemalis*), a songbird that has been used extensively in studies of evolutionary and behavioral endocrinology (Ketterson et al., 2009; Ketterson and Nolan, 1992). Juncos are thought to have rapidly diverged into several phenotypically distinct subspecies since the last glaciation (Mila et al., 2007), and we compare two subspecies that differ in a number of T-mediated traits. White-winged juncos (*Junco hyemalis aikeni*), which breed only in the Black Hills of South Dakota, are significantly larger, more ornamented, and more aggressive than the Carolina subspecies (*Junco hyemalis carolinensis*) (Bergeon Burns et al., 2013; Nolan et al., 2002), which breed in the Appalachian mountains of Virginia. The subspecies do not differ in average T levels when sampled in the field (Bergeon Burns et al., 2013) or when sampled in a common aviary environment 30 min after standardized HPG axis activation (Bergeon Burns et al., 2014). Despite these similar T levels measured in prior research, captive VA males appear to be more sensitive to LH stimulation of the gonad (more LH receptor mRNA), and they also may be more sensitive to negative feedback at the top of the HPG axis (more hypothalamic AR mRNA), compared to SD males (Bergeon Burns et al., 2014). These findings led us to hypothesize that the populations may differ in temporal regulation of T secretion, despite not differing significantly in the magnitude of circulating T at the sampling points used in earlier research. Critically, individual male juncos are also repeatable in how much T they produce in response to exogenous injection of GnRH (Jawor et al., 2006), and this individual variation is remarkably similar to the amount of T produced in response to a standardized LH injection (Bergeon Burns et al., 2014). Thus, our past work suggested that individual differences in T production may lie primarily downstream of this LH signal (i.e. at the level of the gonad).

In this study, we examined gonadal tissues from free-living male juncos that were sampled on their breeding territories in the early spring, as well as captive males held in a common aviary environment. We measured T levels and expression of genes whose products have central roles in gonadal steroidogenesis. We asked whether the two phenotypically divergent populations varied in gonadal gene expression in the wild, and whether any differences persisted in a common garden. We investigated which genes, if any, predict individual differences in T levels. We predicted that males with higher T would have greater gonadal gene expression for these steroidogenic enzymes. We further predicted that males from the larger, more ornamented, and more aggressive population would have a greater molecular capacity to produce T, and that when the HPG axis was stimulated, these males would elevate T more rapidly and sustain that elevation for a longer period of time.

Material and methods

Study 1: Testosterone and gonadal gene expression in the field

Male juncos were captured on their breeding territories in the spring near Custer, South Dakota ("SD," 43°46′N 103°36′W, $n = 17$, dates: 14 to 22 May 2010) and Mountain Lake, Virginia ("VA," 37°22′N, 80°32′W, $n = 17$, dates: 1 May to 5 June 2010), as described in prior publications on aggressive behavior and neural gene expression in these same birds (Bergeon Burns et al., 2014; Rosvall et al., 2012a). All animals experienced a 6 min simulated territorial intrusion ("STI") by a live same-sex conspecific between 0600 and 1200 local time. Males were captured shortly thereafter (4.7 ± 0.8 min after STI), and immediately killed by overdose of isoflurane, followed by decapitation. Trunk blood was collected and stored on ice in the field, and plasma was stored at −20 °C. Gonads were dissected from the body using RNAse-free techniques, flash frozen on dry ice, and stored at −80 °C until processing. Exact breeding stage was not known for most males, but males were reliably defending a territory, and in some cases we observed females building nests and incubating, suggesting that the timing of collection was after territory establishment, in the early- to mid- breeding period. Enlarged gonads were typical of full reproductive condition (see below).

The short STIs are not expected to have affected T levels because juncos do not elevate T in response to STIs under these conditions (Rosvall et al., 2012b, 2014). In addition, appreciable transcription of all but immediate early genes is thought to require more time (Herdegen and Leah, 1998), and it is thought to require at least 60 min for appropriate upstream hormonal stimuli to affect our genes of interest (LaVoie and King, 2009). Accordingly, we anticipate that expression of genes measured here represents a close approximation of each male's constitutive expression prior to the short STI, rather than a genomic response to the STI itself. Consistent with this view, latency to capture was not