Evaluating testosterone as a phenotypic integrator: from tissues to individuals to species

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Hormones have the potential to bring about rapid phenotypic change; however, they are highly conserved over millions of years of evolution. Here, we examine the evolution of hormone-mediated phenotypes, and the extent to which regulation is achieved via independence or integration of the many components of endocrine systems. We focus on the sex steroid testosterone (T), its cognate receptor (androgen receptor) and related endocrine components. We pose predictions about the mechanisms underlying phenotypic integration, including coordinated sensitivity to T within and among tissues and along the HPG axis. We then assess these predictions with case studies from wild birds, asking whether gene expression related to androgenic signaling naturally co-varies among individuals in ways that would promote phenotypic integration. Finally, we review how mechanisms of integration and independence vary over developmental or evolutionary time, and we find limited support for integration.

Keywords: integrated phenotype, testosterone, evolutionary constraint, evolutionary potential, androgen receptor, auto-regulation

1. Integration, independence, and testosterone

Phenotypic integration occurs when molecular, physiological, morphological, behavioral, or life history traits co-vary (Pigliucci 2003; Murren 2012). Integration is considered adaptive because co-expressed trait combinations can yield higher relative fitness than the alternative, when traits are decoupled (Sinervo and Svensson 1998; McGlothlin and Ketterson 2008). Thus, 'integrated phenotypes' typically refer to cases in which we observe strong correlations between two or more components of the phenotype that work well together– for example, courtship displays, aggression, and gametogenesis during social competition, versus offspring provisioning and reduced territorial aggression during parental care (Ketterson and Nolan 1999; Wingfield et al. 2001). There are many ways to conceptualize the degree of integration, all of which generally reflect patterns of covariation among phenotypic traits (Klingenberg 2008; Armbruster et al. 2014).

Hormones are a physiological component of the integrated phenotype, and they also may generate integration (Zera et al. 2007; Jaillais and Chory 2011; Martin et al. 2011; Hau et al. 2016). Much of the work on phenotypic integration in vertebrates has examined the hormone testosterone (T), focusing on higher vs. lower levels in circulation, both naturally and experimentally (Hau & Wingfield 2011; Ketterson & Nolan 1999; Wingfield et al. 2001). T has been linked with various socially or sexually selected traits (Sinervo and Svensson 1998; Kempenaers et al. 2008), and in driving the expression of such traits under different social or ecological contexts, T can mediate life history trade-offs (McGlothlin and Ketterson 2008). Although T levels fluctuate temporally (Williams 2008), there is also evidence that individual variation in T is repeatable (Pelletier et al. 2003; While et al. 2010, but see Jawor et al. 2007, Pavitt et al. 2015) and that T levels are heritable (Kempenaers et al. 2008), setting the stage for this hormone to play a role in phenotypic evolution (Hau and Goymann 2015; Cox et al. 2016).

Importantly for behavioral ecologists, T is also relatively inexpensive and straightforward to assay. It can be sampled from the blood of wild individuals that readily return to life as freely behaving animals shortly thereafter. However, the focus on T *signal*, rather than the many other endocrine components that influence the effects of T, oversimplifies its integrative power as a mediator of phenotypic change. This highlights an important evolutionary consideration, which is often ignored in behavioral ecology: the signaling capacity of a molecule is effective only within the system that generates and receives it (Dufty et al. 2002; Ball and Balthazart 2008; Kempenaers et al. 2008). As a standalone molecule, T is highly conserved (in fact, identical) in structure over millions of years of evolution (Adkins-Regan 2005). There is no gene for T; rather, the evolution of T-mediated traits depends upon the mechanisms that produce, regulate, and respond to T, i.e., the entire endocrine phenotype. Critically, our understanding of how the androgenic signaling system evolves in wild animals is limited.

Roughly a decade ago, two models were proposed to conceptualize how selection shapes the evolution of hormone-mediated traits, each with two non-mutually exclusive hypotheses. Hau (2007) proposed that hormone-mediated traits evolve via (a) *evolutionary constraint*, whereby life history trait combinations, hormone levels, and other components of endocrine systems are tightly linked and co-evolve in a conserved fashion across species, or (b) they evolve via *evolutionary potential*, in which hormone

signal (i.e. T levels) can evolve independently from hormone receptors, metabolism, conversion, co-factors, and so on. Ketterson et al. (2009) describes two related models as (a) *phenotypic integration*, the tight connection between hormone signals and hormonally mediated traits, and (b) *phenotypic independence*, the flexible uncoupling of these traits, which is proposed to be mediated by tissue-level differences in sensitivity to hormones. Ketterson and colleagues view integration as both a potential driver and constrainer of evolutionary change. For instance, integration could hasten phenotypic change if coordinated changes across many traits are adaptive in new environments, such that a single change in T levels would produce sweeping consequences. However, if the constellation of traits tightly integrated by T is no longer beneficial in a new environment, then divergence would be constrained. In contrast, one or another trait can become de-coupled from the otherwise integrated phenotype, for instance via changes in androgen sensitivity in a particular tissue, which would then facilitate change. Both Hau and Ketterson et al. consider these models as part of a continuum, a perspective that we carry forward below.

Here, we evaluate evidence for these hypotheses of independence vs. integration, focusing on putatively co-regulated components of the androgenic signaling system. We examine the expression patterns of many genes across multiple tissues, from non-model organisms in their natural environment. We begin by laying out predictions of phenotypic integration at the tissue level (§3), i.e. how endocrine mechanisms could generate organismal integration. We evaluate these predictions with novel network analyses assessing the degree of integration in datasets drawn from our own research on free-living birds (§4) in which we have measured many components of the endocrine phenotype across multiple tissues, in the same individuals (Rosvall et al. 2012; Bergeon Burns et al. 2013; Rosvall et al. 2013; Bergeon Burns et al. 2014; Bentz et al. 2019). We then examine how integration varies across scales: within or among individuals, between the sexes, and among species (§5). We close by highlighting challenges and next steps for applying this framework to evolutionary endocrinology (§6), to further build connections between behavioral ecology and evolutionary endocrinology in our shared endeavor to understand the evolution of integrated phenotypes.

2. Unpacking mechanisms of testosterone-mediated integration

T is produced in the gonad(s) and transported in the bloodstream to neural and peripheral tissues. In doing so, T is pleiotropic – it can coordinate expression of multiple traits via downstream effects on many organs, tissues, or cell populations (Lema and Kitano 2013). For instance, during the breeding season for many vertebrates, T influences suites of social and reproductive behaviors by activating key areas of the brain, as well as the muscles, gonads, and other peripheral tissues (Wingfield et al. 2001).

T-mediated traits are regulated by an endocrine system that has many names, including the androgenic signaling cascade (Fuxjager and Schuppe 2018), T production and response pathway (Hau and Wingfield 2011; Rosvall et al. 2016), T regime (Kempenaers et al. 2008), hormone control system (Adkins-Regan 2008), and endocrine phenotype (Cox et al. 2016), all of which generally refer to a network of interacting components that includes binding globulins, enzymes for steroid synthesis

and metabolism, T metabolites, hormone receptors, cofactors, response elements and their collective effect on downstream gene expression. Many consider T to be permissive, such that the precise amount in circulation may not be relevant, except to the degree that it exceeds a threshold above which it is likely to activate downstream effects (Hews and Moore 1997; Hau and Goymann 2015). Others find a dose-dependent relationship between T and phenotypic traits (Bhasin et al. 2001), or non-linear relationships. Thus, the phrase 'T-mediated traits' is not limited to traits that co-vary with circulating T levels per se, but it more accurately conveys that trait expression is influenced by one or more components of this system (see Ball and Balthazart 2008; Williams 2008).

Nuclear androgen receptors (AR) are one component of this system generating phenotypic variation. Once T or its more potent metabolite 5α-dihydrotestosterone (DHT) binds to AR, the steroid-receptor complex acts as a transcription factor, targeting the promotors of specific genes and regulating their expression (Hunter et al. 2018). AR expression is auto-regulated by T, and T can induce or suppress AR transcription in a tissue-specific manner (Nastiuk and Clayton 1994; Bagamasbad and Denver 2011; Hunter et al. 2018). T can also be converted into 17β-estradiol (E_2) via the enzyme aromatase (AROM). In fact, many of the well-studied behavioral effects of T are mediated via E_2, which can bind to estrogen receptors (ER) or act via rapid activation of secondary messenger systems (Remage-Healey et al. 2018). T can also be synthesized locally from androgen precursors in many tissues, including the brain (London et al. 2009), adrenals (Soma et al. 2015), and other peripheral tissues (Schmidt et al. 2008). To the degree that locally produced T affects traits of interest via downstream conversion, binding, and/or transcription, selection can act on local tissue-specific control of hormonally mediated traits *independently* of levels of T in circulation.

The mechanistic significance of these many components and their target-specific effects is far from new to molecular endocrinology (Dufty et al. 2002; Schmidt et al. 2008). Until recently (Rosvall et al. 2012; Cox et al. 2016; Fuxjager and Schuppe 2018), however, behavioral ecology and evolutionary endocrinology have largely overlooked quantification of other components of androgenic signaling systems (i.e. beyond circulating T levels), despite discussion that one (or more) components may be mechanistic drivers of evolutionary change (Hau 2007; Ketterson 2009). Filling this knowledge gap will require that we examine multiple components of the endocrine phenotype and their interactions with morphological, behavioral and life history traits, both within populations and between species. After all, it is the system as a whole that produces the collective organismal traits visible to natural selection, influencing the probability of who lives or dies, who breeds and how much.

3. Predictions of phenotypic integration at the tissue level

As summarized above, most work on T-mediated phenotypes focuses on connecting endocrine traits (here, T levels) with non-endocrine traits, such as morphology, physiology, and behavior, as they relate to life history trade-offs. Here we shift our focus under the skin, to describe non-mutually exclusive endocrine mechanisms that ought to influence the degree of phenotypic integration. We largely conceptualize full integration as *positive* co-variation among components of the endocrine system. However, we also expect negative co-variation due to the potential

for autoregulation, which can generate either positive or negative co-variation depending on abundance and the balance between positive vs. negative feedback (Hunter et al. 2018). Like Hau and Ketterson and colleagues, we view integration as a continuum, ranging from strong correlations among all parameters (integration), to only a few strong connections, to essentially none (independence).

One prediction of integration is that AR expression is linked among tissues, leading to the coordinated expression of multiple traits. Although not typically stated, such co-regulation most parsimoniously depends upon *cross-tissue integration of sensitivity to T*. Most simply, this would occur if some individuals have relatively higher levels of AR and others have lower levels of AR across tissues (e.g. brain, muscles, gonads; for an analogous perspective on glucocorticoids, see Lattin et al. 2015). This prediction need not be limited to AR, and could apply to other endocrine traits such as enzymatic steroid conversion (AROM, 5α-reductase), other steroid receptors (ER), and co-factors, among others. Relative abundance of AR could be manifest at the level of gene expression (mRNA), protein abundance, number of AR-immunoreactive neurons, and so on, although we do not distinguish among these possibilities here. Presumably having more AR across tissues would facilitate organismal integration, promoting for example, greater spermatogenesis, athleticism or activity, sexual behavior, and courtship, alongside higher T levels. At the other end of the spectrum, AR expression across tissues may be completely independent, with AR in each tissue varying independently of other tissues and T levels in circulation (Hunter et al. 2018).

A second prediction regarding the mechanisms underlying integration involves *endocrine cascades,* such that functionally related components are regulated in concert. For T, this would occur along the hypothalamic-pituitary-gonadal (HPG) axis, where an individual with high T levels also produces more GnRH from the hypothalamus, has more abundant GnRH-receptors in the pituitary, secretes more LH, and has more abundant LH-receptors in the gonad. This cascade-level integration might be extended further, with 'high responders' also having stronger negative feedback responses along multiple tiers of the HPG axis or greater metabolism of T in the liver.

A third prediction relates to whether different components of the T-production-response system are *integrated within a tissue* (sensu evolutionary constraint, Hau 2007). Although within-tissue integration or independence does not inherently link (or de-couple) multiple organismal traits across tissues, it nevertheless tells us how animals might regulate tissues independently from T, and it therefore has important implications for phenotypic integration. Full integration would occur when local steroidogenic capabilities (synthesis, conversion, and degradation) and hormone sensitivity (binding globulins, receptors, and cofactors) are coordinated within a tissue. Under this scenario, for instance, individuals with high AR within a particular brain area may also have higher AROM (converts T to E_2), higher 5α-reductase (converts T to DHT), higher expression of specific co-factors, and may even activate more genes or to a greater degree, compared to other individuals with comparatively lower levels of these many components. Alternatively, these components may be independently regulated, perhaps allowing animals to shunt more hormone towards one pathway than another (e.g. androgenic vs. estrogenic, synthesis vs. metabolism, etc.).

4. Case Studies from wild birds and insights into integration vs. independence

Data that bear on co-variation of endocrine components are limited, and so we begin by reanalyzing mostly published data to evaluate cross-tissue and cross-cascade integration with T, capitalizing on data that we generated with colleagues over the last 10 years in multiple projects. Using these data, we begin to test the hypothesis that individual variation in T co-varies with other endocrine traits, including hormone receptors and enzymes that metabolize T, across multiple neural and peripheral tissues. We focus on individual variation because individual differences are the raw material of evolution (Williams 2008) and heritable individual differences are proportional to the potential for natural selection (reviewed in Cox et al. 2016). We employ a network approach where each node represents a trait and each edge represents Spearman's rank correlation coefficient (ρ) between two traits (Wilkins et al. 2015), thereby making it robust to outliers. We analyze connectivity within networks (ρ_{sum}), which is the sum of the absolute value of ρ among all traits, i.e. the sum of connection strengths among nodes, divided by the number of possible connections to standardize for maximum potential integration. ρ_{sum} is akin to intramodular connectivity in weighted gene co-expression network analysis (Zhang and Horvath 2005). We also calculate $T\rho_{sum}$, which reflects connectivity between just T and all other traits. We visualize networks with cytoscape (Shannon et al. 2003), only displaying strong correlations ($|\rho| \geq 0.7$). There are many ways to quantify network properties and we do not assert this approach is the only way; rather, these analyses are meant to serve as a spring-board for understanding how integration may or may not be generated and how it can vary over different timescales, from the developmental to the evolutionary.

In doing so, these examples begin to address whether and how integration changes across breeding stages within an animal's lifetime (Bentz et al. 2019), how males and females may differ in endocrine integration (Rosvall et al. 2012, 2013), and ultimately, how the degree of integration itself may change as populations and species diverge (Bergeon Burns et al. 2013; Bergeon Burns et al. 2014). With one exception (Figure 1a,b), these data are previously published and we simply re-analyze them in a network framework. One analysis includes new data on AR gene expression generated from additional tissues from animals sampled for another project (Bentz et al. 2019). For an overview of animal collection and gene expression methods, see SI Methods.

First, we examined how the degree of phenotypic integration varies across breeding stages, focusing on T levels and AR gene expression in neural and peripheral tissues in female tree swallows (*Tachycineta bicolor*) (Figure 1a,b). Aggression in this system is an important predictor of a female's ability to acquire a nesting cavity (Rosvall 2008), with females engaging in acrobatic aerial chases, which can escalate to physical aggression (Stutchbury and Robertson 1987). Ovarian T production capabilities and circulating T levels are high during territorial establishment but lower during incubation and chick-rearing (George and Rosvall 2018; Bentz et al. 2019), and experimental work demonstrates that T promotes female aggression and mediates trade-offs with maternal behavior (Rosvall 2013a). Here, we measured AR gene expression across 3 peripheral tissues (ovary, spleen, muscle; Betnz et al. 2019) and 4 macro-dissected neural tissues (hypothalamus [hypo], ventromedial telencephalon [VmT], hippocampus [HpC], and hindbrain [HB]) using females that we collected during territorial establishment or incubation (Bentz et al. 2019; George and Rosvall unpublished data). Three clear patterns emerge from these data: (1) T levels are not consistently correlated with AR

gene expression across tissues, indicating tissue-specific patterns of autoregulation, (2) AR gene expression in one tissue generally varies independently of AR gene expression in other tissues, although (3) patterns of cross-tissue integration vary from one breeding stage to the next. For instance, during territorial establishment, positive co-variation occurs between T, AR in pectoral muscle, AR in VmT (a socially responsive brain area), and AR in gonad (Figure 1a). It is tempting to interpret this as an integrated set of phenotypes that works well together (i.e. higher T alongside higher AR in neural and peripheral tissues that affect social aggression during territorial establishment), but we do not have behavioral data to address this directly. On the whole, these data demonstrate that cross-tissue AR expression can become more integrated, as environmental and physiological selection pressures shift from territorial establishment to incubation within the breeding season.

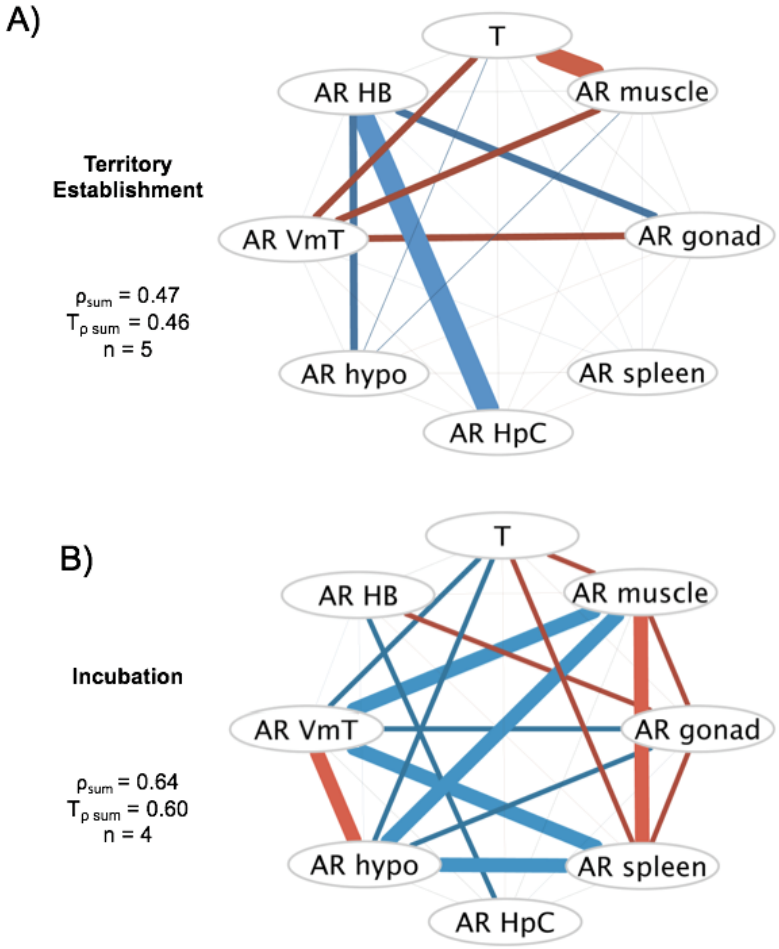

Figure 1: Integration of T and AR gene expression across tissues and breeding stages. in female tree swallows (*Tachycineta bicolor*) during a) territorial establishment and b) incubation. Edge color indicates the direction of the correlation (positive = red, negative = blue) and line thickness indicates the strength (edge threshold is $|\rho| \geq 0.7$. ρ_{sum} represents the sum of $|\rho|$ among nodes, divided by the number of possible node connections to standardize for maximum potential integration. $T\rho_{sum}$ reflects the sum of $|\rho|$ between T and all other nodes, divided by the number of possible node connections

to T. Abbreviations: T = testosterone, AR = androgen receptor, VmT = ventromedial telencephalon, hypo = hypothalamus, HpC = hippocampus, HB = hindbrain.

Next, we evaluated evidence for cross-tissue integration in the brain, and how it varies between males and females. These data come from dark-eyed juncos of the Carolina subspecies (*Junco hyemalis carolinensis*). All individuals were captured and collected on their territories during the early-to-mid breeding season (see SI Methods). Females were in the incubation stage and males were in breeding condition, as evidenced by enlarged gonads. We measured AR, ER, and AROM gene expression in VmT, HYPO, and right posterior telencephalon (PT) because these brain areas have been implicated in the regulation of aggressive behavior (Rosvall et al. 2012). Network analyses show that across neural tissues, AR, ER, and AROM gene expression are not integrated with T for either sex (Figure 2a,b). There is some suggestion of within-tissue integration for both sexes (e.g. ER, AR, AROM in HYPO) and more so for males in the PT and VmT. Weaker integration in females supports the prediction that the independence of endocrine components could be an adaptive mechanism to minimize the costs of T (Rosvall 2013b). For females, a negative correlation of AROM in the VmT with ER in the PT suggests some negative feedback relating to E_2, although without E_2 measurements, this is untested. On the whole, these results suggest independence across neural tissues, but integration within neural tissues for both sexes.

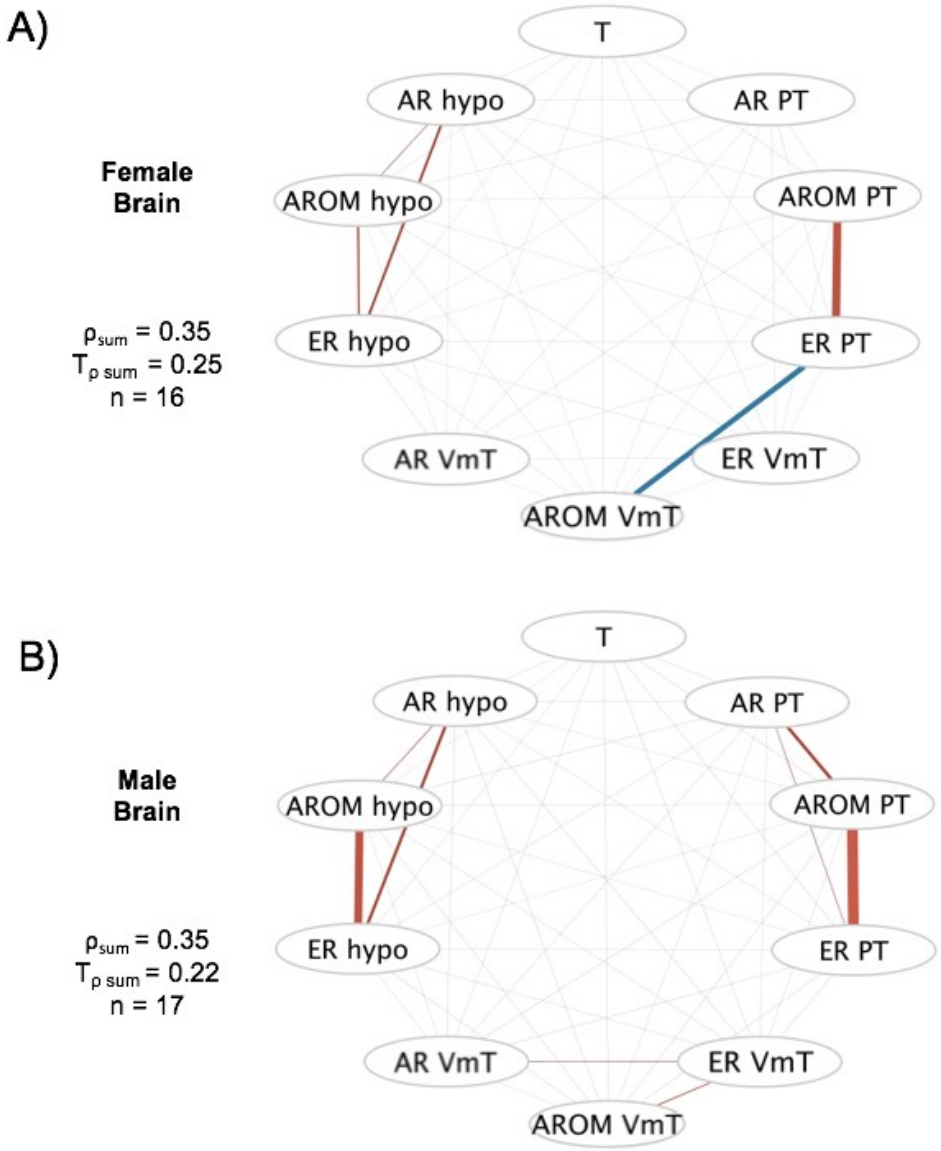

Figure 2: Integration of AR, ER, and AROM gene expression within and across neural tissues in Carolina dark-eyed junco (*Junco hyemalis carolinensis*) a) females and b) males. Edge color indicates the direction of the correlation (positive = red, negative = blue) and line thickness indicates the strength (edge threshold is $|\rho| \geq 0.7$). ρ_{sum} represents the sum of the absolute value of $|\rho|$ among nodes, divided by the number of possible node connections to standardize for maximum potential integration. $T\rho_{sum}$ reflects the sum of the absolute value of $|\rho|$ between T and all other nodes, divided by the number of possible node connections to T. Abbreviations: T = testosterone, AR = androgen receptor, ER = estrogen receptor, AROM = aromatase, VmT = ventromedial telencephalon, hypo = hypothalamus, PT = posterior telencephalon.

Finally, we compared two subspecies of junco that differ in multiple T-mediated traits, to explore how integration across the HPG axis may change over evolutionary time. We compared male dark-eyed juncos of the Carolina subspecies (*J. h. carolinensis*), with males of the larger, more aggressive, and more ornamented

congener, the white-winged junco (*J. h. aikeni*), which are thought to have diverged around the last glacial maximum (Friis et al. 2016). White-winged males elevate T more rapidly and for a longer period of time following HPG axis stimulation (Rosvall et al. 2016). They also have more white on their tail feathers, a sexually selected trait, and are more aggressive in the number of flyovers during an STI, compared to Carolina males (Bergeon Burns et al. 2014). In a previous study, we sampled these two subspecies in a common aviary environment that used a photo-stimulatory day length to mimic early spring conditions, when males have enlarged gonads (Bergeon Burns et al. 2014; Rosvall et al. 2013; see SI Methods). When individuals were injected with GnRH and LH (counterbalanced and separated by a few days), they produced almost identical levels of T each time. The amount of LH secreted in response to GnRH challenges was unrelated to this gonadal output of T, and stimulated levels were largely unrelated to baseline levels, perhaps consistent with the pulsatile secretion of LH. LH-receptor gene expression in the testis (LHR gonad) and hypothalamic gene expression at the top of the HPG axis (AR hypo, AROM hypo) also were uncorrelated with individual differences in T production. Later work showed that testicular steroidogenic gene expression was a significant predictor of T output in this system (Rosvall et al. 2016), highlighting that the gonadal tier of this endocrine cascade (i.e. the testes) was integrated with T production. When we combine these data in a network framework, this reveals only limited evidence for integration along the HPG axis in either subspecies (Figure 3a,b), though future work could examine more complex, non-linear relationships.

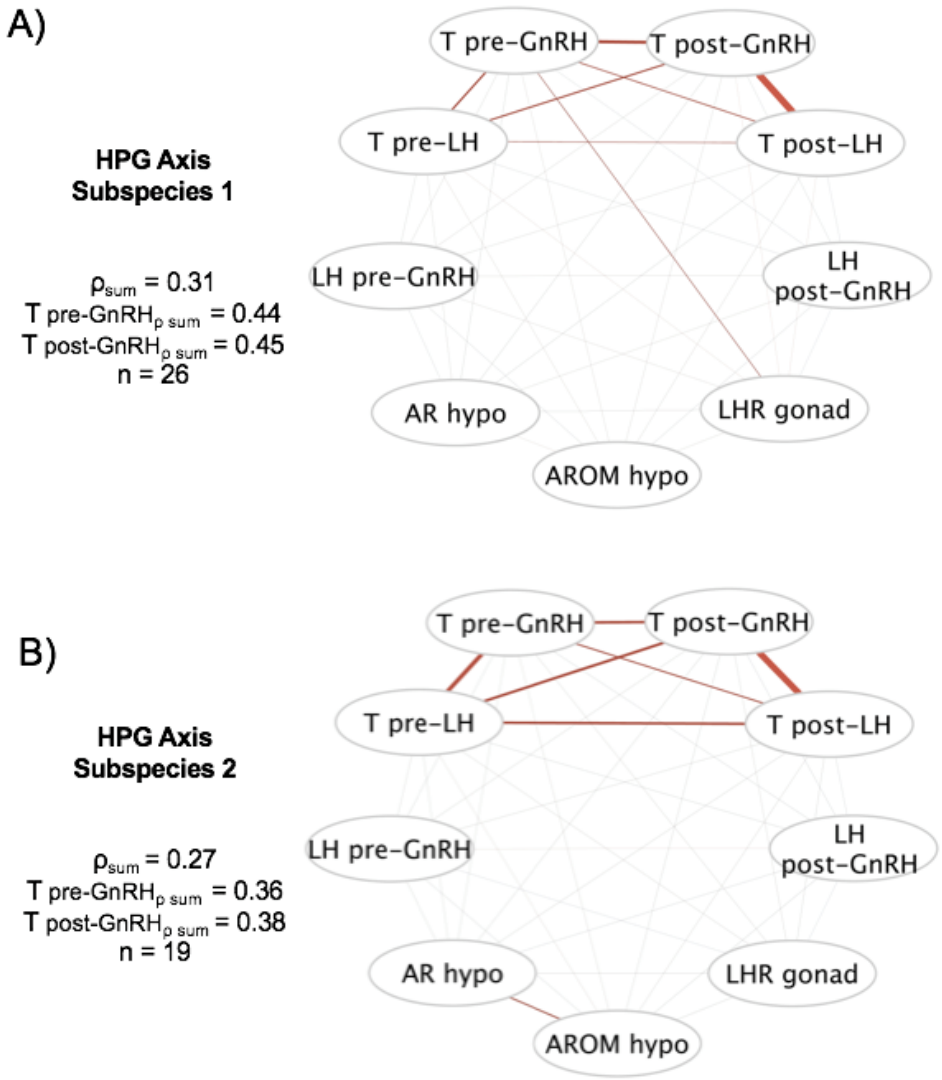

Figure 3: Integration of HPG axis in male juncos from a) the Carolina subspecies (*J. h. carolinensis*) and b) white-winged subspecies (*J. h. aikeni*), which are more aggressive and elevate T more rapidly following HPG axis stimulation. Edge color indicates the direction of the correlation (positive = red, negative = blue) and line thickness indicates the strength (edge threshold is $|\rho| \geq 0.7$). ρ_{sum} represents the sum of the absolute value of $|\rho|$ among nodes, divided by the number of possible node connections to standardize for maximum potential integration. $T\rho_{sum}$ reflects the sum of the absolute value of $|\rho|$ between T and all other nodes, divided by the number of possible node connections to T. Abbreviations: T = testosterone, AR = androgen receptor, hypo = hypothalamus, LH = luteinizing hormone, GnRH = gonadotropin-releasing hormone.

These novel network analyses provide limited support for integration of T with components of the endocrine phenotype across tissues and endocrine cascades. Interestingly, we found some support for within-tissue integration of endocrine phenotypes in the brain (Figure 2a,b).

5. Scales of integration: individuals to sexes to species

5.1 Integration and independence within a lifetime

Whether and how individual variation in endocrine parameters relates to other organismal traits (e.g. behavior, morphology) is a key question in evolutionary endocrinology (Ball and Balthazart 2008; Kempenaers et al. 2008; Williams 2008; Rosvall et al. 2012). Research linking individual variation in baseline T levels with behavior, however, has been met with limited success (Adkins-Regan 2005), perhaps due to the inherently flexible secretion of T (Kempenaers et al. 2008). Experimental stimulation of the HPG axis with exogenous GnRH injections has mitigated these concerns by flooding the HPG axis with GnRH and quantifying an individual's maximal T output after a standardized waiting period. Results are nonetheless mixed: T levels produced in response to GnRH injections are correlated with suites of T-mediated traits, including territorial aggression, parental care, and ornamentation in dark-eyed juncos (McGlothlin et al. 2008), but they are not correlated with parenting or aggression in Northern cardinals (*Cardinalis cardinalis*) (DeVries et al. 2012). T responses to GnRH challenge also track morph-related differences in T-mediated traits in male white-throated sparrows (*Zonotrichia albicollis*) (Spinney et al. 2006) and side-blotched lizards (*Uta stansburiana*) (Mills et al. 2008), but average T responses to GnRH do not differ among morphs in male red-backed fairy-wrens (*Malarus melanocephalus*) (Barron et al. 2015) or Gouldian finches (*Erythrura gouldiae*) (Cain and Pryke 2017).Thus, the capacity to elevate T levels does not necessarily predict organismal trait integration among individuals within species.

For decades, it has been clear that T-mediated traits differ even among individuals with putatively equal amounts of T in circulation (Grunt & Young 1952), with researchers pointing to other (non-T) components of androgenic signaling system (Ball and Balthazart 2008). More recently, inter-individual differences in behavior have been correlated with variation in measures of neural sensitivity to T or its metabolites (Goodson et al. 2012; Rosvall et al. 2012; Horton et al. 2014). These patterns often differ among brain areas (Trainor et al. 2006), indirectly suggesting that tissue-level sensitivity to sex steroids is not fully integrated across behaviorally relevant brain areas, similar to what we find in both tree swallows (Figure 1a,b) and dark-eyed juncos (Figure 1c,d).

T also may have different effects on individuals at different times, suggesting plasticity in the degree of integration. One interesting example comes from the red-backed fairy wren, in which T affects a different set of integrated traits in young (typically dull in plumage) vs. old (typically bright) males. Bright vs. dull plumage males differ in many social-reproductive behaviors, though young bright males do not share the full suite of behaviors seen in older bright males (Karubian 2002; Webster et al. 2008; Dowling and Webster 2017). T implants administered to young males also induce molt into the full bright plumage (Lindsay et al. 2011) but older males molt into bright plumage despite low T levels in circulation. Morphs also do not differ in their T production potential (Barron et al. 2015). These patterns not only suggest that T plays some role in inducing molt into bright plumage and coordinating other components of the integrated phenotype, but they also imply that: (1) the specific combination of T-sensitive traits may vary with age and (2) this effect might be mediated via age- and/or

morph-related differences in how tissues respond to T. Together, these patterns suggest that cross-tissue integration of AR expression may vary across and individual's lifetime, although this is an empirical question ripe for testing.

Seasonal comparisons likewise present compelling evidence for plasticity in the integrating potential of T. For example, aggression and T co-vary in the breeding season, but are decoupled in the non-breeding season when T levels are low (Demas et al. 2007). Some studies suggest that T implants or AR blockage may affect traits in different ways during breeding vs. non-breeding life history stages as well (e.g. Smith et al. 1997; Sperry et al. 2010). Seasonal patterning of AR and AROM gene expression also varies among brain tissues and in relation to T (Canoine et al. 2007; Wacker et al. 2010), allowing at least semi-independent regulation of the effects of T across the body. Likewise, our assessment of AR gene expression across neural and peripheral tissues in female tree swallows (Figure 1a,b) suggests that integration is flexible and can change over different breeding stages.

5.2 Sex differences in integration and independence from T

Male and female T levels are typically correlated among species, suggesting that female T secretion is shaped by correlated responses to selection acting on males (Møller et al. 2005; Mank 2007; Goymann and Wingfield 2014), but the optimal level of T secretion may differ for each sex (Ketterson et al. 2005). Females also have all components of the androgenic signaling system (Staub and De Beer 1997), and they may experience selection to decouple certain traits from the effects of circulating T, which could be achieved by decreasing T sensitivity in some tissues but not others, thereby facilitating behavioral insensitivity to T at relevant life history stages such as parental care (Lynn 2008; Sperry et al. 2010).

Past research provides some support for sex-specific patterns of covariation in phenotypic traits, though not universally so. For example, in both male and female dark-eyed juncos, the ability to produce T (i.e. hormonal response to GnRH injection) predicts individual differences in territorial aggression (McGlothlin et al. 2007; Cain and Ketterson 2012). However, baseline T levels only predict aggression in males in this system, whereas neural sensitivity to sex steroids predicts aggression in both sexes (Rosvall et al. 2012). Similarly, in the White's skink (*Egernia whitii*), T secretion predicts aggression in males but not in females (While et al. 2010). In masked boobies (S*ula dactylatra*), however, boldness, melanization, and T levels were more strongly inter-correlated in females than males (Fargallo et al. 2014). Similarly, Maruska and Fernald (2010), working on the peripheral auditory system in cichlids (*Astatotilapia burtoni*), report greater co-variation of circulating androgens and AR, ER, and AROM in females compared to males. These examples suggest that there may be sex differences in the mechanisms underlying one or another trait; however, it is less clear whether one sex is generally more integrated than the other or whether the sexes generate integrated phenotypes in different ways, both of which are key questions for future research.

Experimental treatment with T is an especially useful test of the hypothesis that T affects suites of traits differently in males and females. T treatment sometimes produces 'male-typical' courtship behaviors and morphological changes in females (Hausberger et al. 1995; Day et al. 2007; Cox et al. 2015; Lindsay et al. 2016), suggesting that sexual dimorphism is due to sex biases in T production. In female brown anoles (*Anolis*

sagrei), phenotypic integration among T-mediated traits including growth, metabolic rate, and dewlap characteristics is minimal in juveniles with low T, but strong in juveniles with experimentally elevated T (Cox et al. 2016). The potential for T to initiate sexual differentiation of multiple traits at critical periods in young individuals implicates the organizational effects of T during development (Adkins-Regan 2008; Cain et al. 2013; Cox et al. 2017; Lofeu et al. 2017) in addition to activating changes to suites of traits in adults. Other research demonstrates sex-specific effects of T on downstream gene activity (Peterson et al. 2013; Peterson et al. 2014; Cox et al. 2017), with greater modularity in genomic responses in females than males (van Nas et al. 2009), consistent with the prediction that T-mediated traits could be more independent in females (Lynn 2008; Rosvall 2013b).

5.3 Integration and independence across species

Evidence linking T secretion and life history traits among species is mixed (Oliveira et al. 2002; Hirschenhauser and Oliveira 2006; Garamszegi et al. 2008; Hau et al. 2010; Goymann and Wingfield 2014; Miles et al. 2018). This is perhaps unsurprising, considering that expression of T-mediated traits depends on so much more than the level of T in circulation. However, few studies examine these multiple endocrine components alongside other organismal traits. In one such species comparison of *Sceloporus* lizards, *S. undulatus* males have blue belly patch signals, are more aggressive, have higher T and also have more hypothalamic AR cell counts than white-patched females, whereas in the closely related *S. virgatus*, both sexes have white patches and similar levels of T, hypothalamic AR, and aggression (Hews et al. 2012). This suggests that the degree of integration may vary among species, in sometimes sex-dependent ways. Similar implications stem from comparisons of two subspecies of white-shouldered fairy-wrens (*M. alboscapulatus*) that differ in female ornamentation: females from the ornamented population are more aggressive and have higher T, whereas males exhibit subspecific differences in ornamentation and aggression, but not T levels in circulation (Enbody et al. 2018).

Further support for evolutionary variation in integration comes from recent studies of the musculo-skeletal system, which can influence the performance of gestural displays. For example, activation of AR in the scapulohumeralis caudalis muscle produces a ‘roll-snap’ courtship display in the golden-collared manakin (*Manacus vitellinus*) (Fuxjager et al. 2017). Critically, AR expression in this wing muscle is associated with motor complexity of courtship displays across manakin species (Fuxjager et al. 2015). In another example in anoles, species that perform high rates of pushup displays and locomotor movements have a greater proportion of bicep nuclei positive for AR expression (Johnson et al. 2018), and there was a moderately significant correlation between species-average T levels and AR expression. Notably, these studies utilized phylogenetic comparative methods, allowing robust linkages between endocrine traits (here, AR) and behavioral traits. An exciting extension could explore how multiple components of androgenic signaling systems evolve independently or in concert to produce morphological and behavioral trait variation across taxa.

Recent examination of AR and cofactor expression across multiple tissues and species provides some of the best data on cross-tissue integration of endocrine

phenotypes. Fuxjager and Schuppe (2018), looking at 4 tissues including liver, pectoralis muscle, eye, and testis in 3 different species, find no consistent pattern of AR expression with SRC1 and NCOR1 cofactor expression within or across tissues, suggesting independence across tissues. For male golden-collared manakins, AR expression levels are similar in the wing muscles and spinal cord but not in the testes (Fuxjager et al. 2015). In male Bornean rock frogs with a foot-flagging display, AR expression in leg muscles and spinal cord were higher than in non-flagging species (Mangiamele et al. 2016). However, there were no AR differences in brain or larynx tissue across species, and no consistent species variation in AR across all tissues. Together, these studies suggest substantial variation in cross-tissue AR integration among species, implying independence of tissue-specific T sensitivity rather than autoregulation of AR and T across tissues.

6. Conclusions and future directions

Testosterone and the traits it mediates have captivated biologists for decades. Nowhere is this more apparent than in the nexus of behavioral ecology with endocrinology, where evolutionary endocrinologists are investigating both proximate and ultimate questions about integrated phenotypes in free-living animals. Our review evaluates previously unmet predictions of this line of work, most critically that varying T levels are associated with other components of the androgenic signaling system across the organism, in ways that would promote phenotypic integration. This sort of 'obligate' integration, in which T coordinates the same traits in the same way across developmental or evolutionary timescales, stands in contrast to the data we review here. We report limited evidence for integration across tissues or along endocrine cascades (e.g. AR integration among neural and peripheral tissues, integration of different components of the HPG axis). We also find that the degree of integration may vary within an animal's lifetime, further supporting the view that multiple components of the complex endocrine phenotype can vary independently and flexibly to bring about behavioral or morphological diversity.

In closing, we propose three initiatives that will help to distill this complexity towards new insight. First, as this special issue highlights, T is among many hormones thought to be phenotypic integrators and drivers of evolutionary change. However, it is an open question whether or how T-mediated integration differs from that of glucocorticoids, insulin-like growth factor, and others. Will these more metabolic hormones that affect life-death functioning yield greater potential for integration than T? Or, is the relative lack of integration across the organism a more common phenomenon shared by many hormones? Limited evidence to date suggests perhaps so (Lattin et al. 2015), but this is another empirical question ripe for testing. Second, experimental work has not sufficiently addressed whether T-mediated integration is indeed adaptive. Considering the degree of cross-tissue independence described above, it may be fruitful to further de-integrate organisms, e.g. via tissue-specific RNAi (Casasa and Moczek 2018) or hormonal manipulations that cannot cross the blood-brain barrier (e.g. AR antagonist bicalutamide, Fuxjager et al. 2013), thereby changing the suite of traits affected by circulating hormones. Finally, the relative lack of within population integration we report here (i.e. among-individual co-variation between components of the endocrine phenotype) stands in contrast to other examples of phenotypic integration

across sexes and species (Hews et al. 2012; Cox et al. 2017; Johnson et al. 2018; Mangiamele et al. 2016). Perhaps by examining linear co-variation, we have missed integration by more complex endocrine components that act more permissively on trait expression (Hews and Moore 1997). The variety of potential relationships between T and phenotypic traits suggests that an absence of linear correlations does not necessarily indicate independence, but rather that integration can be complex, and multiple models should be tested in the future. Another potential reason for this mismatch between correlative individual comparisons (within-group) and categorical evolutionary comparisons (across-group) is that current trait values of standing phenotypic variation differ from patterns that emerge over larger developmental or evolutionary scales. Functional individual variation in endocrine systems has received less attention than comparisons among groups, despite calls for more studies over a decade ago (Williams 2008). As these data continue to accumulate across a broad phylogenetic scale, our understanding of endocrine mechanisms and their role in phenotypic evolution will only improve, making this an exciting time for evolutionary endocrinology.

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