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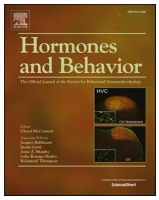

Evolution of hormone-phenotype couplings and hormone-genome interactions

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ABSTRACT

When selection favors a new relationship between a cue and a hormonally mediated response, adaptation can proceed by altering the hormonal signal that is produced or by altering the phenotypic response to the hormonal signal. The field of evolutionary endocrinology has made considerable progress toward understanding the evolution of hormonal signals, but we know much less about the evolution of hormone-phenotype couplings, particularly at the hormone-genome interface. We briefly review and classify the mechanisms through which these hormone-phenotype couplings likely evolve, using androgens and their receptors and genomic response elements to illustrate our view. We then present two empirical studies of hormone-phenotype couplings, one rooted in evolutionary quantitative genetics and another in comparative transcriptomics, each focused on the regulation of sexually dimorphic phenotypes by testosterone (T) in the brown anole lizard (*Anolis sagrei*). First, we illustrate the potential for hormone-phenotype couplings to evolve by showing that coloration of the dewlap (an ornament used in behavioral displays) exhibits significant heritability in its responsiveness to T, implying that anoles harbor genetic variance in the architecture of hormonal pleiotropy. Second, we combine T manipulations with analyses of the liver transcriptome to ask whether and how statistical methods for characterizing modules of co-expressed genes and in silico techniques for identifying androgen response elements (AREs) can improve our understanding of hormone-genome interactions. We conclude by emphasizing important avenues for future work at the hormone-genome interface, particularly those conducted in a comparative evolutionary framework.

1. Evolution of hormone-phenotype couplings

Hormones can be conceptualized as intermediate signals that allow organisms to translate a variety of intrinsic physiological or extrinsic environmental cues into appropriate phenotypic responses. We use the term "cues" broadly to refer to a diversity of inputs (e.g., sex, age, season, abiotic environment, social environment) that can occur over a variety of temporal scales (e.g., minutes, hours, seasons, developmental stages), just as we use "phenotypic responses" to encompass everything from transient behaviors to permanent developmental outcomes. When selection favors a new relationship between a cue and phenotypic response (Fig. 1A), adaptation can proceed through genetic change in the coupling of cue to hormonal signal (Fig. 1B), or in the coupling of hormonal signal to phenotypic response (Fig. 1C-E). In other words, selection should favor a change in the strength or timing of the hormonal signal that the cue elicits, or in the sensitivity of target tissues and genes to that hormonal signal (Ketterson et al., 2009). Here, we focus on the second of these two possibilities – the evolution of hormone-phenotype couplings – by using androgens and their signaling pathways as a framework in which to explore emerging evolutionary questions involving hormone-genome interactions.

Due to the relative ease with which circulating hormone levels can be quantified, the field of evolutionary endocrinology has focused primarily on the evolution of hormonal signals rather than hormone-phenotype couplings. For example, circulating hormone levels have been analyzed extensively as traits in phenotypic selection analyses (Bonier et al., 2009; John-Alder et al., 2009; McGlothlin et al., 2010; Ouyang et al., 2013; Ouyang et al., 2011; Patterson et al., 2014), quantitative genetic analyses (Béziers et al., 2019; Cox et al., 2016; Iserbyt et al., 2015; Jenkins et al., 2014; Pavitt et al., 2014; Stedman

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et al., 2017), and comparative phylogenetic analyses (Goymann et al., 2018; Husak and Lovern, 2014; Møller et al., 2005; Vitousek et al., 2018). Of course, circulating hormone levels are but one feature of complex endocrine networks that also include binding globulins for hormone transport, enzymes for hormone metabolism, receptors for hormone signaling, response elements in DNA that establish the genomic targets of hormone receptors, and co-factors that mediate the hormonal regulation of gene expression (Cox, 2020; Denver et al., 2009; Fuxjager and Schuppe, 2018; Ketterson et al., 2009; Lipshutz et al., 2019). Recent work has underscored this complexity by highlighting the

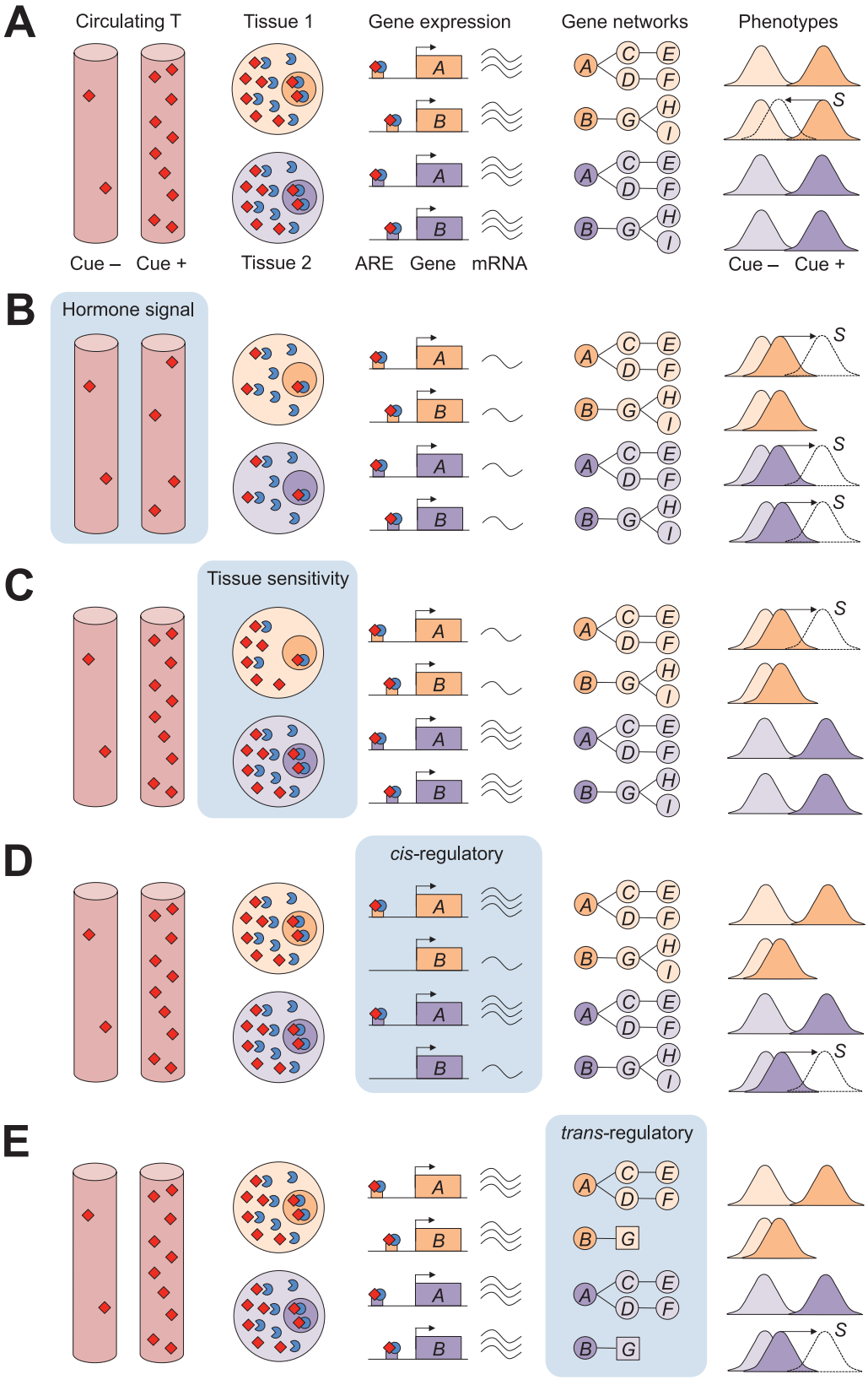


Fig. 1. Mechanisms for the evolutionary decoupling of hormonally mediated phenotypes. (A) Secretion of testosterone (T, red diamonds) into circulation increases in the presence of a cue (e.g., sex, season, social environment), leading to increased binding of androgen receptors (AR, blue pies) in the cells of target tissues (orange and purple circles). Bound AR enters the nucleus and interacts with androgen response elements (AREs) in the regulatory regions of target genes (*A* and *B*), leading to increased mRNA transcription. Products of these *cis*-regulated targe genes influence the expression of downstream genes (*C*—*I*), creating *trans*-regulated gene networks that ultimately lead to differences in phenotypic expression in the presence versus absence of the cue. Suppose that selection (*S*) now favors a decrease in the responsiveness of a phenotype to this cue (dashed outline represents this new favored phenotypic distribution, colored shapes show the distribution produced by the existing architecture). (B) Altering the coupling between the cue and hormonal signal lowers circulating T and ultimately decreases the expression of multiple phenotypes in multiple tissues, potentially disrupting many phenotypes from their fitness optima. (C) Altering the responsiveness of a target tissue (shown here as a reduction in AR density, but potentially also including changes in enzymes or transcription cofactors) increases the specificity of phenotypic change, but it may disrupt other phenotypes regulated by the same tissue. (D) Uncoupling *cis*-regulated target genes (shown here as the loss of an ARE for gene *B*) increases the specificity of phenotypic change, but it may disrupt expression of the target gene in other tissues. (E) Modifying components of downstream gene networks (shown here as a modification of gene *G* that disrupts the network and limits its phenotypic effects) increases the specificity of phenotypic change, but it may disrupt network expression in other tissues. Effects of hormonal pleiotropy generate selection (*S*) against "off-target" phenotypic changes in panels B-E.

role of binding globulins in shaping hormone availability (Breuner and Orchinik, 2002; Breuner et al., 2013; Malisch and Breuner, 2010), by calling attention to the limitations of inferences about natural selection on hormone levels that are inherently plastic or condition-dependent (Bonier and Martin, 2016; Dantzer et al., 2016; Malkoc et al., 2021), and by quantifying the scope of hormone production in response to a stimulus when studying repeatability (Casto and Edwards, 2021; Hau and Goymann, 2015; Jawor et al., 2006; Taff et al., 2018), heritability (Bairos-Novak et al., 2017; Béziers et al., 2019; Stedman et al., 2017), experimental evolution (Evans et al., 2006), and natural or sexual selection (McGlothlin et al., 2010; Patterson et al., 2014). Nonetheless, these studies have focused largely on circulating hormonal signals and their availability to target tissues, rather than on the downstream couplings between hormonal signals and the phenotypes they regulate.

When considering how phenotypes evolve, there is a growing appreciation that changes in hormone-phenotype couplings may often be of greater importance than upstream changes in the production of a hormonal signal (Bergeon Burns et al., 2014; Cox, 2020; Fuxjager and Schuppe, 2018; Hau, 2007; Husak and Lovern, 2014; Lipshutz et al., 2019). This view is based in part on the rationale that circulating hormone levels must simultaneously provide appropriate contextual signals for each of the many phenotypes that they regulate. The ability of a single hormone to regulate multiple phenotypes is often referred to as hormonal pleiotropy, a concept that has generated considerable discussion about whether and how hormonal signaling networks constrain or facilitate evolutionary change (Adkins-Regan, 2008; Bourg et al., 2019; Cox, 2020; Dantzer and Swanson, 2017; Finch and Rose, 1995; Flatt et al., 2005; Hau, 2007; Ketterson et al., 2009; Ketterson and Nolan, 1999; Lema, 2014; McGlothlin and Ketterson, 2008; Schuppe and Fuxjager, 2019). Due to hormonal pleiotropy, changes in the sensitivity of individual phenotypes to stable hormonal signals should generally provide a more favorable path for phenotypic evolution than changes to the hormonal signal itself, which could potentially disrupt numerous other phenotypes from their fitness optima (Fig. 1B). This should be especially true when the existing degree of hormonal pleiotropy is high and when the phenotypic effects of hormones are not easily reversible.

For peptide hormones that are encoded by genes, hormonal pleiotropy is directly analogous to genetic pleiotropy (i.e., one gene influencing multiple phenotypes). However, for steroid hormones that are biosynthesized from cholesterol (e.g., testosterone, our focus in this paper), pleiotropy is mediated by the combination of the hormone ligand and its nuclear receptor (i.e., androgen receptor, AR), which acts as a transcription factor and is encoded in the genome, making the receptor a potential target of evolution. The AR and other related steroid receptors are thought to be evolutionarily conserved across vertebrates and, while this is true of some key receptor domains (e.g., AR domains that bind hormone ligands, AR domains that bind response elements in the DNA of target genes), others regions of the same receptor can exhibit considerable evolutionary lability (e.g., AR domains that interface with co-regulators of gene expression) (Schuppe et al., 2020). Across bird species, there is some evidence that changes in the hinge domain of the AR have occurred in lineages characterized by sexual selection for elaborate courtship displays that involve androgen signaling (Schuppe et al., 2020). Changes in receptor structure and function therefore present an intriguing and understudied facet of the evolution of hormone-phenotype couplings. However, because such changes presumably influence hormonal signaling throughout the organism, they may provide a mechanism broadly analogous to upstream changes in circulating hormone levels themselves, allowing species to "turn up" (or "turn down") steroid signaling (Schuppe et al., 2020). As noted above, precise changes in the coupling of individual phenotypes to hormonal signals may often present a more favorable evolutionary path than wholesale changes to hormone production or receptor function.

If changes in hormone-phenotype couplings are relatively simple to achieve, evolutionarily speaking, then phenotypes have high potential for "hormonal independence" (Ketterson et al., 2009) and the pleiotropic architecture of hormonal regulation should yield considerable "evolutionary potential" (Hau, 2007). Ketterson et al. (2009) envisioned this process as one in which target tissues "unplug from" (or "plug into") hormonal signals by evolving reduced (or increased) sensitivity to these signals through reduced (or increased) receptor expression in target tissues (Fig. 1C). In support of this view, species differences in AR expression in muscle tissue are associated with the evolution of androgen-mediated pushup displays and locomotor movements in anole lizards (Johnson et al., 2018), acrobatic courtship displays and associated wing movements in manakins (Fuxjager et al., 2015), territorial drumming in woodpeckers (Schuppe and Fuxjager, 2019), and "foot flagging" displays in frogs (Anderson et al., 2021; Mangiamele et al., 2016). Likewise, a decrease in male-male aggression and corresponding loss of aggressive color signals between two closely related species of *Sceloporus* lizards is associated with lower levels of AR expression in brain regions that mediate aggression (Hews et al., 2012). However, across two *Junco* songbird subspecies, males of the lineage with reduced size, ornamentation, and aggression actually exhibit higher levels of transcript for AR in brain regions that mediate aggression (Bergeon Burns et al., 2014; Bergeon Burns et al., 2013; Rosvall et al., 2012). In this case, intraspecific divergence in T-mediated phenotypes may have occurred primarily through changes in gene networks that regulate the gonadal production of T, or through changes to estrogen-signaling pathways that are activated following the aromatization of T to E_2 (Rosvall et al., 2016a; Rosvall et al., 2016b).

In some species, individual variation in the expression of AR is positively correlated across different tissues (Lattin et al., 2015), suggesting evolutionary constraint in the tissue-specificity of hormonal sensitivity. However, other studies have found that interspecific variation in AR expression is not strongly correlated across different tissues (Fuxjager et al., 2015), suggesting evolutionary potential whereby change in hormonal sensitivity can be restricted to those phenotypes mediated by a particular cell or tissue type. In tree swallows, upstream genes encoding enzymes for testosterone biosynthesis are highly tissue-specific in their expression (primarily in gonads), whereas intermediate genes encoding enzymes for the conversion of testosterone to other potent androgens and estrogens are expressed more broadly across multiple tissues (brain, gonads, liver), and downstream genes encoding androgen and estrogen receptors are highly expressed across many tissues (brain, gonads, liver, spleen, muscle) (Bentz et al., 2019). Therefore, tissue specificity may generally decrease along the pathway from upstream hormone biosynthesis to downstream signaling. An additional layer of cell or tissue specificity in the availability of various transcriptional cofactors may help to further modulate the extent to which hormone-receptor complexes influence the expression of individual genes (Fuxjager and Schuppe, 2018). While cofactor expression can differ by sex to facilitate the evolution of sexual dimorphism (Duncan and Carruth, 2011) and evolve in tissue-specific fashion across species (Fuxjager and Schuppe, 2018), the extent to which such changes underlie the evolution of hormonally mediated phenotypes remains largely unexplored. However, evidence supports the idea that transcriptional cofactors may be more evolutionarily constrained than other components of androgen signaling pathways, potentially because these cofactors must also mediate the transcriptional responses of other signaling pathways (Schuppe and Fuxjager, 2019).

Alternatively, adaptation may proceed by altering the nucleotide sequences that define hormone response elements, such that individual target genes (rather than target tissues) can "unplug from" (or "plug into") a hormonal signal (Fig. 1D). For example, androgens exert their genomic effects by binding AR in the cytosol and causing its translocation to the nucleus, where the activated hormone-receptor complex recruits cofactors to promote, enhance, or repress the expression of target genes that contain androgen response elements (AREs) in their regulatory regions (Cox, 2020; Fuxjager and Schuppe, 2018). AREs are short DNA sequences that typically consist of a pair of direct or inverted repeats separated by 3 variable (n) nucleotides (e.g., ARE consensus

motif with inverted repeats: 5′-AGAACAnnnTGTTCT-3′; ARE "selective" motif with direct repeats: 5′-AGAACAnnnAGAACA-3′), and shorter "half" motifs can also bind AR (Denayer et al., 2010; Filho et al., 2019; Starr et al., 2017; Tewari et al., 2012). Substitutions at some nucleotide positions in the ARE (or proto-ARE) will prevent (or enable) AR binding, but substitutions at other nucleotide positions may have only minor stimulatory or inhibitory effects on AR binding, such that a variety of phenotypic effects on hormonal sensitivity of gene expression are theoretically possible. Estimates of the number of unique binding regions for AR in vertebrate genomes range from thousands to tens of thousands, and many genes that are differentially expressed in response to androgens have proximate AR binding sites, implying a considerable amount of *cis* regulation (reviewed by Cox, 2020). While this suggests abundant evolutionary potential for the coupling or decoupling of individual genes to or from hormonal signals, few studies have attempted to link phenotypic evolution to genomic changes in hormone response elements (Frankl-Vilches et al., 2015; Fuxjager et al., 2016; Fuxjager and Schuppe, 2018), and those that have done so have relied primarily on in silico analyses rather than direct empirical approaches that characterize AR interactions with the genome (see Section 5, below).

Conceptually, we find it useful to distinguish among three classes of mechanism for the evolution of hormone-phenotype couplings (Fig. 1C-E). First, as discussed above, target *tissues* (or cell types) can increase or decrease their sensitivity to a hormonal signal though evolutionary changes in expression of hormone receptors, enzymes for hormone metabolism, transcription cofactors that mediate their genomic effects, or signaling molecules that mediate their non-genomic effects (Fig. 1C). This class of mechanism theoretically enhances evolutionary potential by altering hormonal sensitivity in tissue-specific fashion, thereby reducing any negative pleiotropic consequences across other tissues. However, it may also present a constraint in the sense that all genes in the target tissue (more precisely, all genes that are typically expressed in that tissue) will experience a similar change in hormonal sensitivity. Second, target *genes* can gain or lose sensitivity in terms of their direct *cis* regulation through evolutionary changes in the nucleotide sequences that establish hormone response elements in their regulatory regions (Fig. 1D). This class of mechanism theoretically increases specificity with respect to the genomic targets of evolutionary change (Wray, 2007), but it may also present a constraint in the sense that these changes will occur across all tissues (again, to the extent that these target genes are expressed in other tissues). Third, target *networks* downstream from genes that are *cis* regulated by hormone receptors can evolve such that the *trans*-regulatory effects of the hormone signal are altered (Fig. 1E). Among other things, this class of mechanism can include changes in the sensitivity of downstream genes to the products of hormonally *cis*-regulated genes as well as changes that alter the products of downstream genes. In theory, such changes can provide even greater phenotypic specificity and thereby mitigate the negative effects of hormonal pleiotropy. Mechanisms in this third category may often prove more difficult to link to hormonal signaling because they occur downstream of canonical endocrine features such as hormone receptors and response elements. Other mechanisms, such as changes in the epigenetic processes that mediate chromatin accessibility (Tewari et al., 2012), are presumably also important for the evolution of AR/ARE interactions but are not explicitly included in Fig. 1.

The evolution of hormone-phenotype couplings is expected to occur through a combination of the mechanisms describe above, but certain phenotypes may be predisposed to one or another of these evolutionary paths. Changes in *tissue* sensitivity to a hormonal signal (Fig. 1C) may be particularly likely to evolve when the tissue is already relatively specialized with respect to the phenotype under selection. For example, in manakin species that produce sound via rapid wing- and roll-snapping during courtship, various flight muscles have evolved increased AR levels, whereas other tissues have not (Fuxjager et al., 2015). Flight muscles that are directly involved in sound production, such as the scapulohumeralis caudalis (SH), also exhibit enhanced transcriptional responsiveness to androgens, relative to flight muscles that are not involved in these behaviors, such as the pectoralis (Fuxjager and Schuppe, 2018). In this example, the potential for deleterious pleiotropic effects of tissue-wide increases in hormonal sensitivity may be relatively low due to the specialized functional role of the SH. For tissues or cell types that coordinate a diverse array of biological processes involving many underlying gene networks (e.g., liver tissue and hepatocytes), changes in overall tissue sensitivity could present greater pleiotropic constraints, potentially favoring downstream changes in the hormonal sensitivity of particular *cis*-regulated genes or *trans*-regulated networks. Distinguishing among different mechanisms for hormone-phenotype couplings (Fig. 1) may therefore provide a useful framework for predicting and interpreting features of endocrine evolution.

Regardless of the specific mechanisms involved (Fig. 1), hormone-phenotype couplings can only evolve if populations harbor heritable variation in the relationship between hormonal signal and phenotypic response (Cox, 2020; Cox et al., 2016). Many studies have characterized genetic variance in circulating hormone levels (for recent reviews, see Cox et al., 2016; Guindre-Parker, 2018), or in the magnitude of a hormonal response to a stimulus (Bairos-Novak et al., 2017; Béziers et al., 2019; Stedman et al., 2017). Others have documented genetic correlations between circulating hormone levels and target phenotypes (Ruuskanen et al., 2016; Schroderus et al., 2010), but such correlations could simply reflect genetic variance in the hormonal signal itself (Dantzer and Swanson, 2017). Therefore, despite growing appreciation of the importance of quantifying individual variation in phenotypic responses to hormones (Bergeon Burns et al., 2014; Bergeon Burns et al., 2013; Casto and Edwards, 2021; Cox et al., 2016; Malkoc et al., 2021; Williams, 2008; Williams, 2012), we still lack empirical data on whether and how this phenotypic variance maps onto underlying genetic variance. This is partly because robust estimates of genetic variance and heritability typically require large sample sizes from controlled breeding experiments or pedigreed wild populations (Cox et al., 2016), and partly because it can be difficult to isolate genetic variance in phenotypic responsiveness to hormones from upstream genetic variance in hormonal signal in the absence of experiments. Therefore, an outstanding question in evolutionary endocrinology concerns the extent to which individual variation in phenotypic responsiveness to a hormonal signal has a genetic basis and can thereby evolve in response to selection. Below, we introduce the regulation of sexual dimorphism by testosterone in *Anolis* lizards as a framework for addressing this question about genetic variance in hormone-phenotype couplings, and for exploring regulatory features of the hormone-genome interface.

2. Testosterone and sexual dimorphism in *Anolis* lizards

The brown anole (*Anolis sagrei*) is a small lizard in which adult males are typically 20–40 % longer and 2–3 times more massive than adult females (Cox and Calsbeek, 2010), and in which T stimulates skeletal growth, mass gain, and metabolic rate (Cox et al., 2015; Cox et al., 2009a; Cox et al., 2009b). Males also exhibit a large and brightly colored dewlap that is extended from the throat in concert with stereotyped behavioral displays (pushups, head bobs) during intrasexual, intersexual, and interspecific communication (Cox et al., 2009b). Relative to males, females have a much smaller dewlap that also differs in some aspects of its coloration (Fig. 2) (Cox et al., 2017a), and females use their dewlap and associated behavioral displays much less frequently in territorial interactions (Reedy et al., 2017). Dewlap size, coloration, and associated behavioral displays are all influenced by T in this species (Fig. 2) (Cox et al., 2015; Cox et al., 2017a; Wittman et al., 2021). We use the androgenic regulation of sexual dimorphism in dewlap size and color as a framework in which to explore genetic variation in hormone-phenotype couplings (Section 3), and we use androgenic regulation of sexual dimorphism in growth and body size as a framework in which to explore hormone-genome interactions by characterizing networks of co-expressed genes in the liver (Section 4) and then examining relationships

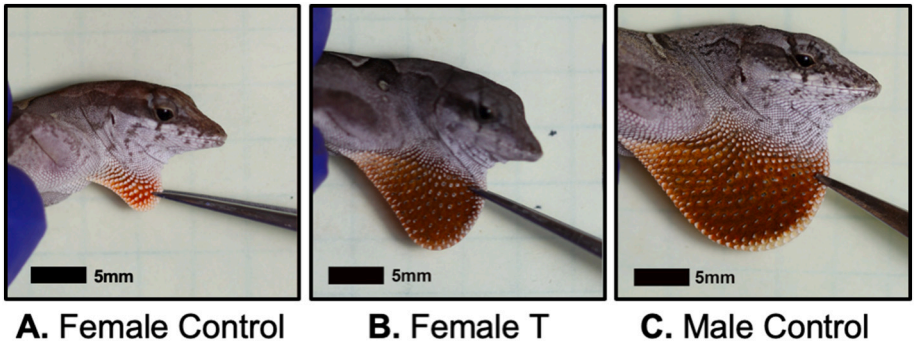


Fig. 2. Representative images of *Anolis sagrei* illustrating dewlap size and color in (A) control female, (B) female treated with testosterone (T) implant, and (C) control male.

between T-mediated gene expression and predicted androgen response elements (Section 5). The experimental data that we re-analyze were originally reported in two studies (Cox et al., 2017b; Wittman et al., 2021) that followed procedures approved by the University of Virginia's Animal Care and Use Committee (protocol 3896).

Sexual dimorphism provides an interesting context for exploring the evolution of hormone-phenotype couplings and hormone-genome interactions because it represents a situation in which two different phenotypes are produced from a genome that is predominantly shared by both sexes. For example, in *A. carolinensis* and related lizards, the sex chromosomes contain only a small fraction of the total genome (Alfioldi et al., 2011; Westfall et al., 2021). Not surprisingly, the vast majority (97 %) of genes that exhibit significant sex differences in hepatic expression in *A. sagrei* during the development of sexual dimorphism in body size map to autosomes in the *A. carolinensis* genome, including all of the genes in growth-regulatory endocrine networks such as the growth hormone/insulin like growth factor (GH/IGF) network (Cox et al., 2017b). Treatment of juvenile females with T stimulates growth and masculinizes patterns of gene expression in the liver, both transcriptome-wide and for key genes in the GH/IGF network, such as growth hormone receptor (*GHR*), insulin-like growth factors (*IGF1, IGF2*), and IGF receptors and binding proteins (Cox et al., 2017b). This suggests that the *development* of sexual dimorphism *within* a species can be conceptualized as a process in which sex differences in hormonal signal (circulating T) are key for coordinating the expression of different phenotypes from the same underlying genome (Fig. 1B). However, the *evolution* of sexual dimorphism *across* species may often reflect changes in the coupling of phenotypes to hormonal signals of sex, age, and season, such as T (Anderson et al., 2021; Cox, 2020; Cox and John-Alder, 2005; Cox et al., 2009a; Fuxjager et al., 2015; Husak and Lovern, 2014; Johnson et al., 2018; Mangiamele et al., 2016).

We focus on a simplified experimental paradigm in which juvenile anoles are treated with exogenous T to generate a sharp contrast between high (extended-release T implant) and low (empty implant) levels of T as a hormonal signal. Details of implant construction and experimental design are provided in the original publications (Cox et al., 2015; Cox et al., 2017b; Wittman et al., 2021). This approach is subject to several caveats. First, enzymes such as 5α-reductase and aromatase can convert T to the potent androgen 5α-dihydrotestosterone (DHT) or the estrogen E_2 upon reaching target cells, so exogenous T can induce both AR and ER signaling. However, in *A. sagrei,* treatment of juveniles with exogenous DHT (which cannot be converted to E_2) stimulates growth and dewlap development in the same manner as exogenous T, indirectly implicating AR signaling as the primary pathway through which T influences these phenotypes (A.E. Walsh, T.N. Wittman, and R.M. Cox, unpublished data). Although genes encoding both AR (*AR*) and ER (*ESR1*) are expressed at similar levels in the *A. sagrei* liver, neither differs in expression with respect to sex, T treatment, or their interaction (Fig. S1A). Moreover, we do not detect any hepatic expression of *CYP19A1,* the gene encoding aromatase (read counts = 0 for all individuals). By contrast, genes encoding 5α-reductase (*SRD5A1-3*) are robustly expressed in the liver and one (*SRD5A3*) is upregulated in response to exogenous T (Fig. S1B). While it is possible that some of the experimental effects we detect are mediated by ER signaling following aromatization of exogenous T to E_2 (the same is true of some natural responses to endogenous T during maturation), our data from liver suggest that local conversion of T to E_2 is negligible.

A second caveat is that, due to sex-linked modifiers or early organizational effects of sex steroids, juvenile males and females may respond differently to the same "activational" hormone signal from exogenous T (Adkins-Regan, 2007). Indeed, males and females often differ in levels of AR, aromatase, and transcriptional cofactors (Cornil et al., 2011; Fuxjager and Schuppe, 2018; Hews et al., 2012), and can also differ in their transcriptome-wide responses to T in some species (Peterson et al., 2013; Peterson et al., 2014). Nonetheless, our work on brown anoles indicates that juvenile females and males respond similarly to exogenous T for a variety of sexually dimorphic phenotypes (Cox et al., 2015), and that treatment of juvenile females with T tends to masculinize gene expression (Cox et al., 2017b) and patterns of underlying genetic covariance for T-mediated traits (Wittman et al., 2021). Therefore, treatment of juvenile anoles with T presents a useful, albeit simplified, experimental framework for exploring basic evolutionary dynamics of hormone-phenotype couplings and hormone-genome interactions.

3. Genetic variance in hormone-phenotype couplings

For evolution to proceed through changes in the coupling between hormonal signal and phenotypic response (i.e., for the architecture of hormonal pleiotropy to evolve), there must be genetic variance in the extent to which individuals exhibit a phenotypic response to a given hormonal signal. Although no study to date has directly addressed this issue, a recent experiment generated a suitable dataset to do so. Wittman et al. (2021) bred 120 *Anolis sagrei* dams and 60 sires with known pedigrees in a paternal half-sibling design, raised 938 of their offspring to 3 months of age (when sexual dimorphism is emerging), then split the progeny from each family into two treatment groups that received either (1) a slow-release T implant, or (2) an empty implant as a control. Five months later, these progeny were measured for snout-vent length, dewlap size, dewlap hue, dewlap saturation, and dewlap brightness, then pedigree information from their parents and grandparents was incorporated into "animal models" (Kruuk, 2004; Wilson et al., 2010) to estimate additive genetic variances and covariances (and their variance-standardized analogues, heritability and genetic correlations) for each trait or trait pair. As expected from previous work, T masculinized females by increasing body size, dewlap size, dewlap hue, and dewlap saturation, by reducing dewlap brightness, and by producing patterns of phenotypic covariance similar to those seen in males (Fig. 2). Previous

work had also shown that male and female anoles naturally differ in many aspects of genetic variance and covariance for these traits, and that between-sex genetic correlations are low for most traits (Cox et al., 2017a; Cox et al., 2017b). Wittman et al. (2021) confirmed these patterns when comparing control males and females, but found that treatment of females with T produced patterns of additive genetic variance and covariance that were distinct from those observed in control females, yet indistinguishable from those found in control males or males treated with T. Between-sex genetic correlations were also significantly higher between females treated with T and either control males or males treated with T (Wittman et al., 2021). Collectively, these results suggest that females and males share most of the same underlying genetic architecture for size and dewlap morphology, but natural sex differences in circulating T levels that emerge during maturation subsequently alter patterns of genetic variance and covariance by modifying the way in which these shared genes are translated into sexually dimorphic phenotypes (Wittman et al., 2021).

One implication of the experiment described above is that brown anoles may harbor significant additive genetic variance in phenotypic responsiveness to T. For example, treatment of females with T altered not only the *phenotypic* means, variances, and covariances for most traits, but also the underlying *additive genetic* components of this phenotypic variance and covariance (Wittman et al., 2021). If we reasonably assume that baseline T levels were uniformly low in juvenile females (Cox et al., 2015), and that any inherent genetic variance in mechanisms that mediate the availability of T (e.g., binding globulins, enzymes for metabolism, rates of clearance and excretion) had relatively minor effects on the chronically elevated levels of exogenous T induced by implants (Cox et al., 2015), then induced differences in hormonal signal between control and T females should be similar across families, aside from any random error attributable to implant construction and T delivery. The fact that an experimentally standardized hormonal signal influenced both phenotypic and *genetic* (co)variance therefore implies that the phenotypic effects of T were dependent upon underlying genetic differences among individuals (Wittman et al., 2021). However, Wittman et al. (2021) did not directly test this hypothesis.

To test for genetic variance in phenotypic responsiveness to T, we reanalyzed data from Wittman et al. (2021) and capitalized on the fact that the manipulation of T in females essentially created two dichotomous "environments" (low T, high T) in which many different "genotypes" (represented by a relationship matrix derived from the pedigree) were expressed as phenotypes. In this context, genetic variance in phenotypic responsiveness to T can be conceptualized as a genotype-by-environment (i.e., family-by-hormone treatment) interaction, and the heritability of responsiveness to T can be estimated from additive genetic variance in the slope of the "reaction norm" across hormone environments (Roff, 1997). Another way of testing this hypothesis is to estimate the genetic correlation for a given trait across hormonal environments (i.e., between control and T females, r_{CT}). Because r_{CT} is estimated from sisters drawn from the same families that differ only in their hormone environment, the null expectation is that r_{CT} should be 1, such that r_{CT} significantly <1 would indicate genetic variance in phenotypic responsiveness to T. To test these predictions, we focused on four components of the dewlap that each respond to T and are also heritable in both the presence and the absence of elevated T: dewlap area, hue, saturation, and brightness. Detailed methods for our analysis are presented as Supplementary Material, and full results are provided in Tables S1-S3.

Fig. 3 plots breeding values for $n = 100$ dams in the experiment across two hormonal "environments" – control (low T) and T implant (high T). Breeding values for each dam are best linear unbiased predictors (BLUPs) of the phenotypes her daughters express in each

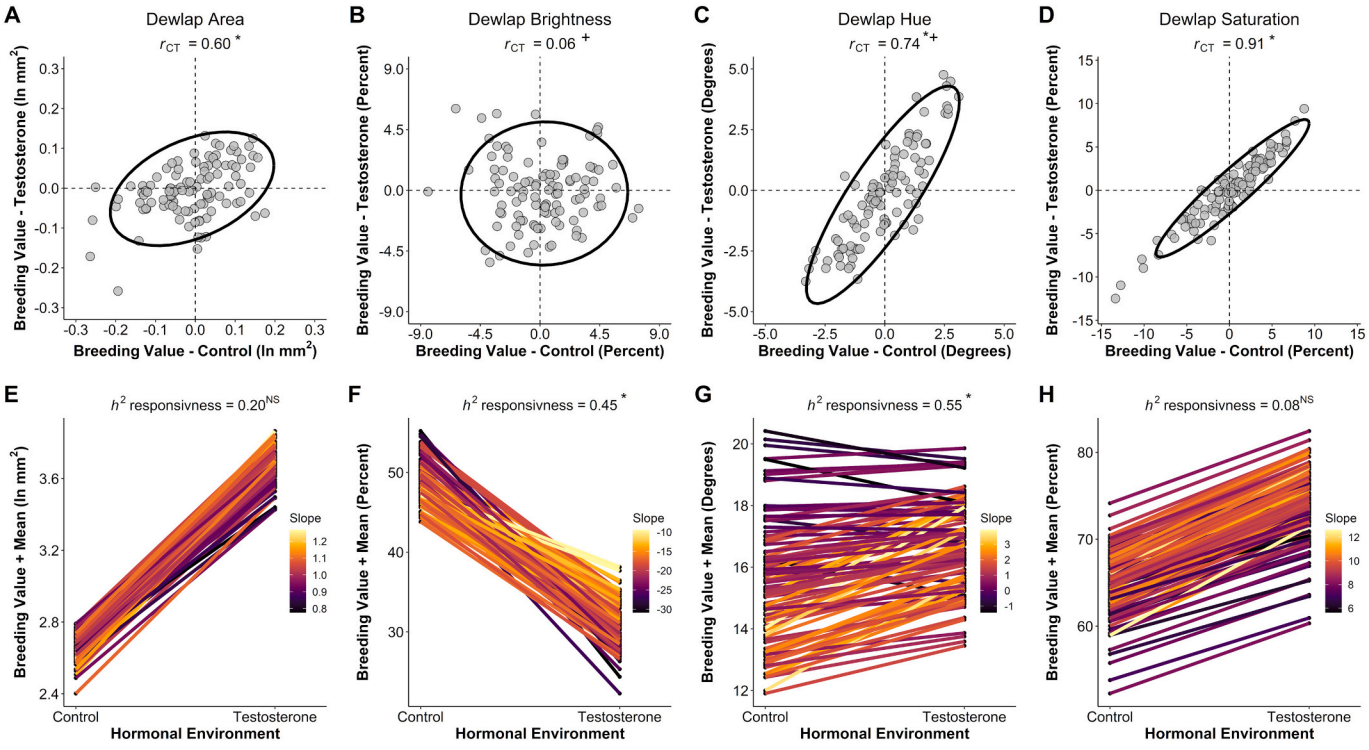


Fig. 3. Genetic variance in phenotypic responsiveness to testosterone (T). Top panels (A-D) show genetic correlations (r_{CT}) between two hormone environments (control, testosterone) for dewlap (A) area, (B) brightness, (C) hue, and (D) saturation. Covariances are illustrated by the breeding values of 100 dams, estimated from the phenotypes expressed by their daughters in each hormone environment. Ellipses are 95 % confidence intervals. Bottom panels (E-H) plot these same breeding values as "reaction norm" slopes across hormone environments, with values in each environment offset by the mean phenotypic effect of T. Colors correspond to relative differences in slope for each phenotype and are not directly comparable across phenotypes. In the absence of genetic variance in phenotypic responsiveness to T, it is expected that $r_{CT} = 1$ (top panels) and that slopes are parallel (bottom panels). Genetic variance in responsiveness to T is evident as $r_{CT} < 1$ (panels B and C), or as crossing slopes with $h^2 > 0$ (panels F and G). See text and Supplementary Material for details on the estimation of breeding values, r_{CT}, and h^2.

hormone environment based on her additive genetic contribution. Breeding values are plotted in two ways to facilitate the visualization of genetic correlations between hormonal environments (r_{CT}, Fig. 3A-D) and of "genotype-by-hormone environment" interactions (Fig. 3E-H). Interestingly, each dewlap phenotype exhibits a different pattern. For dewlap area, T has a pronounced phenotypic effect that slightly weakens the genetic correlation between hormone environments, but not significantly below the expected value of 1 ($r_{CT} = 0.60 \pm 0.31$ SEM; Table S1; Fig. 3A). Though phenotypic responsiveness of dewlap area to T varies slightly across families (Fig. 3E), it is not significantly heritable ($h^2 = 0.20 \pm 0.16$; Tables S2-S3). For dewlap brightness, T has a similarly pronounced effect on the phenotype, but in this case the genetic correlation between hormone environments is virtually eliminated ($r_{CT} = 0.06 \pm 0.38$; Table S1; Fig. 3B) by the wholesale re-ranking of breeding values across hormone environments (i.e., crossing reaction-norm slopes; Fig. 3F), which also manifests as significant heritability of phenotypic responsiveness to T ($h^2 = 0.45 \pm 0.17$; Tables S2-S3). Dewlap hue also exhibits significant heritability in its responsiveness to T ($h^2 = 0.55 \pm 0.23$; Tables S2-S3) and a significant (albeit much smaller) reduction in its genetic correlation between hormone environments ($r_{CT} = 0.74 \pm 0.15$; Table S1; Fig. 3C), despite having a relatively small mean phenotypic effect of T (Fig. 3G). Whereas T increases breeding values for hue in many dams, it decreases breeding values in others (Fig. 3G). Finally, for dewlap saturation, the genetic correlation between hormone environments is close to 1 ($r_{CT} = 0.91 \pm 0.20$; Table S1; Fig. 3D). Although T has a large phenotypic effect on saturation, this effect is highly consistent across dams (i.e., parallel slopes; Fig. 3H), corresponding to low heritability in responsiveness to T ($h^2 = 0.08 \pm 0.18$; Tables S2-S3).

Collectively, our reanalysis of data from Wittman et al. (2021) supports the hypothesis that anoles harbor additive genetic variance in phenotypic responsiveness to hormonal signals, particularly for dewlap hue and brightness. Although female anoles also harbor genetic variance in dewlap area and saturation (as do males; Cox et al., 2017a; Wittman et al., 2021), both of which are phenotypically responsive to T (Fig. 3), we see no evidence of genetic variance in phenotypic responsiveness to T for these traits (i.e., no evidence for genotype-by-hormone environment interactions). Our results imply that there is standing genetic variance in the hormone-phenotype coupling between T and both brightness and hue of the dewlap, such that selection could, in principle, lead to an evolutionary increase or decrease in the extent to which T influences the expression of these traits. Of course, such selection would also (perhaps primarily) occur in males, where additional genetic and environmental variance in circulating T levels (Cox et al., 2016) would likely interact with genetic variance in phenotypic responsiveness to T to influence trait expression. It is also important to note that selection likely does not act directly on the slope of the reaction norm linking dewlap phenotypes across hormone environments, but on the trait values that are produced in a given hormone environment (sex). However, traits such as dewlap hue and brightness offer insight into how selection for enhanced (or reduced) sexual dimorphism could produce an evolutionary response via hormonal regulation. For example, if selection favors the elaboration of existing sexual dimorphism in dewlap brightness, then genotypes with high breeding values in the control (female) environment and low breeding values in the T (male) environment should be favored, thereby selecting for genotypes represented by purple lines in Fig. 3F. If sufficiently strong and consistent, such selection could erode genetic variance in hormonal responsiveness to T, producing patterns similar to those seen for dewlap area and saturation (i.e., parallel slopes in Fig. 3E and H). Hue, which is the least sexually dimorphic and T-responsive component of dewlap color, nonetheless appears to harbor standing genetic variance in phenotypic responsiveness that could eliminate or even reverse the current pattern of sexual dimorphism in which males have slightly higher (redder) dewlap hue than females (i.e., by favoring genotypes represented by purple lines in Fig. 3G).

As in previous studies (Cox et al., 2017a, 2017b; Wittman et al., 2021), we find that dewlap hue and brightness are genetically correlated ($r_G = 0.90 \pm 0.23$; Table S3; Fig. 4A). Interestingly, we also find that *responsiveness* of brightness to T is genetically correlated with *responsiveness* of hue to T ($r_G = 0.70 \pm 0.27$; Table S3; Fig. 4B) and with the *responsiveness* of dewlap area to T ($r_G = -0.74 \pm 0.36$; Table S3). It is unclear whether this occurs because dewlap hue and brightness are relatively independent traits with different underlying genetic architectures that each respond similarly to T, or non-independent readouts of the same underlying genetic pathway(s) involved in color, but the fact that the responsiveness of brightness and area (color and size) are also

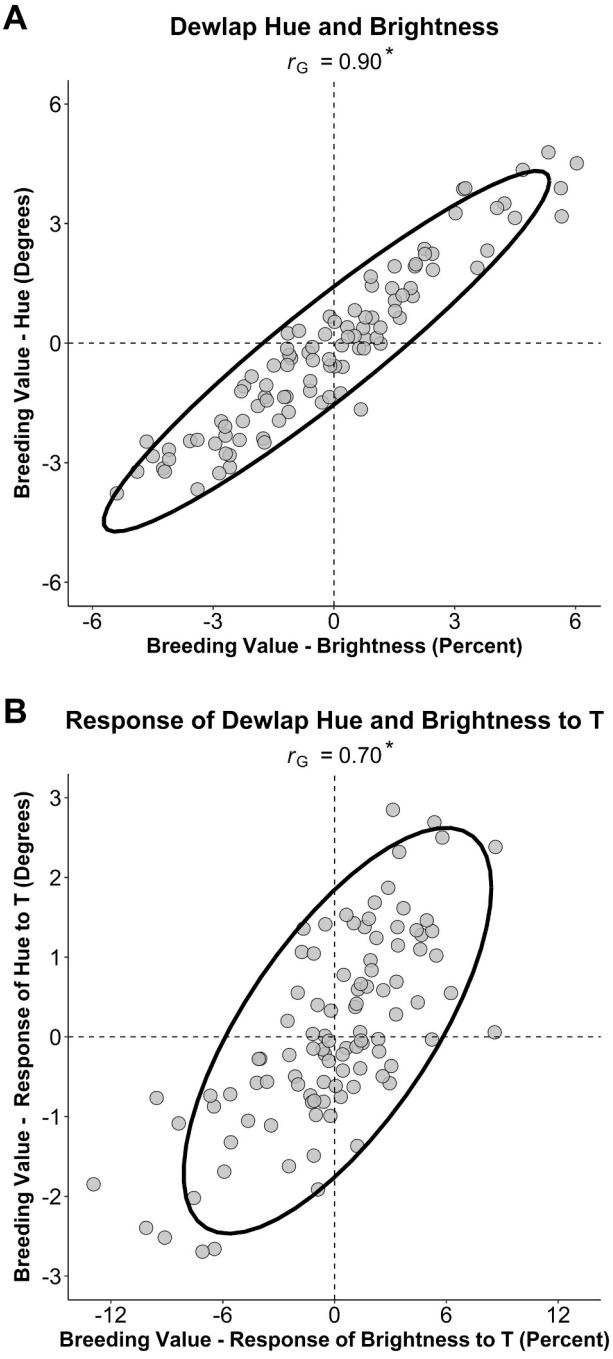


Fig. 4. Genetic correlations between (A) dewlap hue and brightness, and (B) phenotypic responsiveness of dewlap hue and brightness to T. Correlations are derived from a multivariate random regression model that simultaneously estimates the intercepts and slopes for each trait across hormone environments, as well as the between-trait correlations in intercepts (estimated intercepts shown for each dam in A) and slopes (estimated slopes shown for each dam in B).

genetically correlated suggests the former. The first possibility would imply genetic variance in upstream aspects of tissue sensitivity to T (e.g., AR expression in dewlap skin; suggesting evolution could proceed as shown in Fig. 1C), such that multiple independent traits respond in similar fashion within a tissue, whereas the second could reflect genetic variance anywhere in the downstream pathway(s) linking T to trait expression (Fig. 1D-E). This underscores the point that our statistical estimates of heritability in phenotypic responsiveness to T do not provide any direct insight into the mechanistic levels at which this genetic variance resides. For example, our results could reflect segregating genetic variance in AR expression (facilitating evolution as in Fig. 1C), availability of transcriptional cofactors, nucleotide motifs that define AREs in *cis*-regulated target genes (Fig. 1D), and/or downstream *trans*-regulated genes and gene networks (Fig. 1E). To explore some of these mechanistic features in greater detail, we turn our attention to the regulation of gene expression networks by T in a different tissue (liver) with implications for another form of sexual dimorphism (growth and body size).

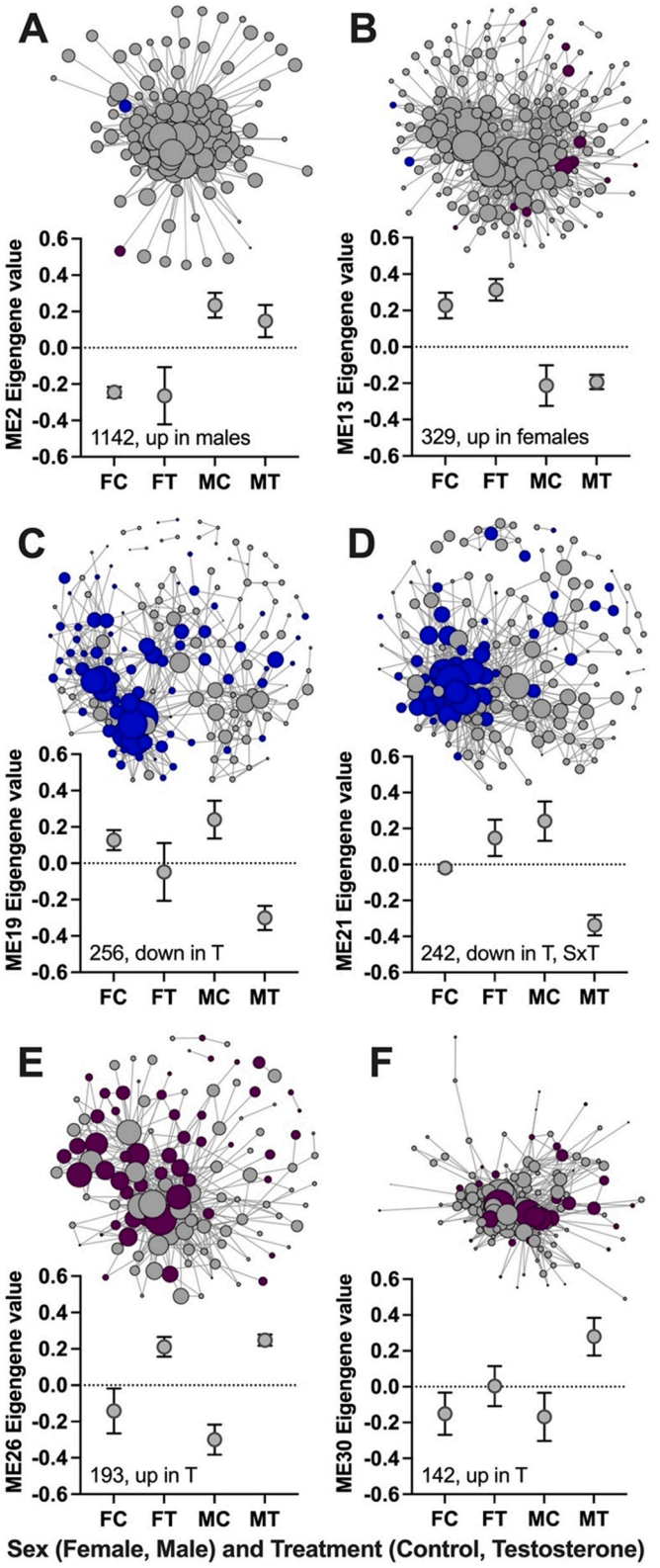
4. Transcriptomes as readouts of hormone-genome interactions

One of the most powerful methods for characterizing the mechanistic basis of hormone-phenotype couplings is by combining hormone manipulations with high-throughput RNA sequencing (RNA-seq) to compare tissue-wide patterns of gene expression in different hormonal environments. With improved methods for de novo transcriptome assembly and increasing availability of annotated genomes for "non-model" species, this approach is now feasible for many organisms familiar to behavioral endocrinologists (e.g., Anderson et al., 2020; Cox et al., 2017b; Frankl-Vilches and Gahr, 2018; Frankl-Vilches et al., 2015; Fuxjager et al., 2016; Peterson et al., 2013; Peterson et al., 2014). A common first step toward biological inference using RNA-seq data is to statistically identify the differentially expressed genes (DEGs) between two comparison groups (e.g., control versus hormone treatment), then use gene ontology enrichment analysis (GO analysis) or related approaches to identify biological pathways that are significantly enriched (i.e., contain more DEGs than expected by chance). For example, analysis of the liver transcriptome during sexual divergence in the growth of brown anoles identified 466 sex-biased genes, and subsequent enrichment analyses identified pathways such as "metabolic processes", "digestion", and "mTOR (mechanistic target of rapamycin) signaling" as significantly enriched for these DEGs (Cox et al., 2017b). One caveat to this approach is that is that care must be taken when interpreting ad hoc explanations for enriched pathways as support for particular a priori hypotheses (e.g., sex-biased expression of the "metabolic processes" pathway is responsible for sexual divergence in growth of *A. sagrei*). One solution could be to identify particular pathways a priori, then test whether the genes in these pathways exhibit predicted patterns of differential expression. For example, Cox et al. (2017b) predicted that sex differences in growth and effects of T on growth are mediated through differential expression of the growth hormone/insulin-like growth factor (GH/IGF) pathway, then found support for this prediction by testing for sex and treatment effects on key upstream genes (e.g., *IGF1*, *IGF2*) as well as the entire GH/IGF gene set.

Another caveat is that DEG analysis takes the rich quantitative data in a transcriptome and reduces it to binary classifications that rely on statistical thresholding to identifying genes of interest. These thresholds are subject to type I-error adjustments to control for the massively parallel nature of statistical hypothesis testing (tests typically involve >10,000 individual genes), which protects against spurious categorization of "significant" DEGs, but also relegates much of the potentially interesting biological signal in a transcriptome to the "uninteresting" bin of non-differentially expressed genes. This concern is exacerbated when the number of biological replicates per group is small, as is common in many RNA-seq studies of non-model organisms (Ingleby et al., 2015). For example, when reanalyzing our published data from livers of juvenile male and female brown anoles treated with T or control implants (n = 3–4 per group; Cox et al., 2017b), we find only 246 significantly sex-biased and 69 significantly T-responsive DEGs (Fig. S1) out of 13,230 expressed genes in the liver (when including sex and treatment as factors, see Supplementary Material).

One way to leverage more of the rich data structure within a transcriptome is through network-based approaches that identify clusters of statistically co-expressed genes, whose holistic properties can then be analyzed in place of individual genes. For example, network analysis of gene expression in muscles used in acrobatic displays by golden collared manakins, compared to zebra finches that lack these behavior displays, revealed numerous modules associated with T treatment and muscle type, thereby facilitating additional insights into the evolution of T-mediated gene expression (Fuxjager et al., 2016). To explore this approach, we applied weighted gene co-expression network analysis (WGCNA, Langfelder and Horvath, 2008) to our published liver transcriptomes from brown anoles, which identified 32 modules ranging in size from 97 to 1524 co-expressed genes (Supplementary Material; Table S2). Two-way ANOVA for each module using eigengene values (i.e., first principal components describing expression variation within that module, see (Velotta et al., 2020) for an analogous approach) reveals large modules of co-expressed genes that are male-biased (e.g., module 2; 1142 genes; sex $F_{1,10} = 22.30$; $P = 0.0008$; Fig. 5A), female-biased (e.g., module 13; 329 genes; sex $F_{1,10} = 33.46$; $P = 0.0002$; Fig. 5B), downregulated by T (e.g., module 19; 256 genes; treatment $F_{1,10} = 12.11$; $P = 0.0059$; Fig. 5C), and upregulated by T (e.g., module 26; 193 genes; treatment $F_{1,10} = 44.12$; $P < 0.0001$; Fig. 5E). Additional modules capture other aspects of sex bias and T responsiveness, including sex-by-treatment interactions (e.g., module 21; 242 genes; interaction $F_{1,10} = 18.92$; $P = 0.0014$; Fig. 5D), albeit at P-values that often fall short of significance thresholds once adjusted for multiple testing (Table S4). One interesting feature of these modules is that T-responsive genes ($-0.5 < \log_2$ fold change < 0.5) tend to be both centrally located and highly connected within T-responsive modules (Fig. 5C-F; Fig. S3). By contrast, the large modules defined primarily by sex-biased expression contain few T-responsive genes, none of which are centrally located (Fig. 5A-B), suggesting widespread sex differences in hepatic transcription that are largely unrelated to chronic elevation of T.

Modules of co-expressed genes are useful in that they identify suites of genes whose expression is likely influenced by T, either directly (*cis*) or indirectly (*trans*), but in ways that are not necessarily captured by traditional DEG analyses involving statistical thresholding or fold-change comparisons. For example, many genes that fall below a $\log_2$ fold-change threshold when compared between treatment groups (gray symbols) are nonetheless highly correlated with T-responsive genes (blue or purple symbols) and occupy central "hub" positions within T-mediated modules (Fig. 5C-F). Some modules also exhibit visible substructure, suggesting the presence of multiple sub-networks that could reflect distinct biological pathways grouped into modules by their shared responsiveness to T (e.g., module 19; Fig. 5C). However, because modules are defined based on statistical patterns of co-expression, it is often unclear whether they correspond to functional biological pathways, or whether they simply reflect the aggregation of genes with similar expression across a variety of pathways, particularly when they are constructed from samples that are heterogeneous with respect to sex and hormone treatment. Perhaps for this reason, GO tests for enrichment of biological pathways based on module membership are uninformative regarding the functional properties of T-responsive modules in brown anole liver (i.e., no GO enrichment for any of the four T-responsive modules in Fig. 5; see Supplementary Material). However, in other systems, GO analyses based on WGCNA module membership have proven more effective at elucidating the biological processes associated with T-responsive gene networks (Fuxjager et al., 2016; Newhouse and Vernasco, 2020). In part, this could reflect the fact that our sample size ($n = 14$) is below the recommended minimum for WGCNA ($n = 15$) and is also heterogeneous with respect to sex. Because the modules we detect



(caption on next column)

Fig. 5. Representative modules from weighted gene co-expression network analysis of the *A. sagrei* liver transcriptome. The top of each panel is a visualization of the module, with individual nodes (genes) represented by circles of size proportional to their connectivity in the module and edges connecting genes with highly correlated expression. Genes are colored based on their $\log_2$ fold change in expression following T treatment (blue = $\log_2$ FC < -0.5, down-regulated by T; purple = $\log_2$ FC > 0.5, up-regulated by T; gray = $-0.5 <$ $\log_2$ FC < 0.5, not regulated by T; see Fig. 6E). Axes below each module show mean ($\pm$ SE) eigengene (first principal component) values for female controls (FC, $n = 3$), females treated with T (FT, $n = 3$), male controls (MC; $n = 4$), and males treated with T (MT, $n = 4$). Text reports the number of genes in each module and significant effects of sex, treatment, and the sex-by-treatment (SxT) interaction (see Table S2). (A-B) Sex-biased modules contain few T-responsive genes, particularly at central positions within each module. T-responsive modules contain many genes that are down-regulated by T (C—D), or upregulated by T (E-F), particularly at central positions within each module (Fig. S3).

are highly structured by effects of sex and testosterone (Table S4), care should be used when conceptualizing these statistically defined modules as analogues of the functional gene networks or signaling pathways that presumably underlie the evolution of hormone-phenotype couplings (Fig. 1).

5. Linking gene expression to hormone response elements

Whether using individual DEGs or co-expressed modules, it is challenging to link the detailed readouts of hormonal gene regulation that emerge from transcriptome data to underlying regulatory features of the genomic architecture, such as androgen response elements (AREs, see Fig. 1). Yet, such approaches are necessary if we are to understand how genetic changes in regulatory regions of target genes and networks facilitate the evolution of hormone-phenotype couplings. Ideally, physical interactions between hormone receptors and specific genomic regions can be directly inferred using chromatin immunoprecipitation followed by microarray or RNA sequencing (Cheung and Kraus, 2010). In the absence of this empirical gold standard, it has been suggested that in silico analysis of the genome may provide a useful first approximation for identifying putative AREs and other hormone response elements (Anderson et al., 2020; Frankl-Vilches et al., 2015; Fuxjager et al., 2016; Fuxjager and Schuppe, 2018; Hale and Parrott, 2020; Rice et al., 2017). To explore this possibility, we combined our liver transcriptome data from brown anoles (*A. sagrei*) with in silico characterization of putative AREs in the genome of a congener (*A. carolinensis*, Alfioldi et al., 2011) to ask whether the genes and modules whose expression is responsive to T exhibit signatures of *cis* regulation via proximate ARE motifs. Our methods for ARE identification involve a variety of assumptions, including the size of the genomic window used to define spatial proximity to an expressed gene (see Supplementary Material for additional details and caveats associated with in silico analysis). Therefore, we explored patterns across several thresholds (5 kb, 25 kb, 100 kb) for defining ARE proximity (Fig. 6; Fig. S4). Because of this, and because we interpret gene expression in our focal species in relation to the genomic architecture of a congener, our approach is likely to be conservative and to underestimate the magnitude of any biological signal.

Although the regulatory effects of AREs depend on many factors (e.g., their location within promoters or introns, their affinity for AR binding due to their nucleotide motifs, the accessibility of local chromatin, their roles as enhancers or repressors of transcription, their density and proximity to the transcriptional start site), the presence and number of proximate AREs are two readily quantifiable features that

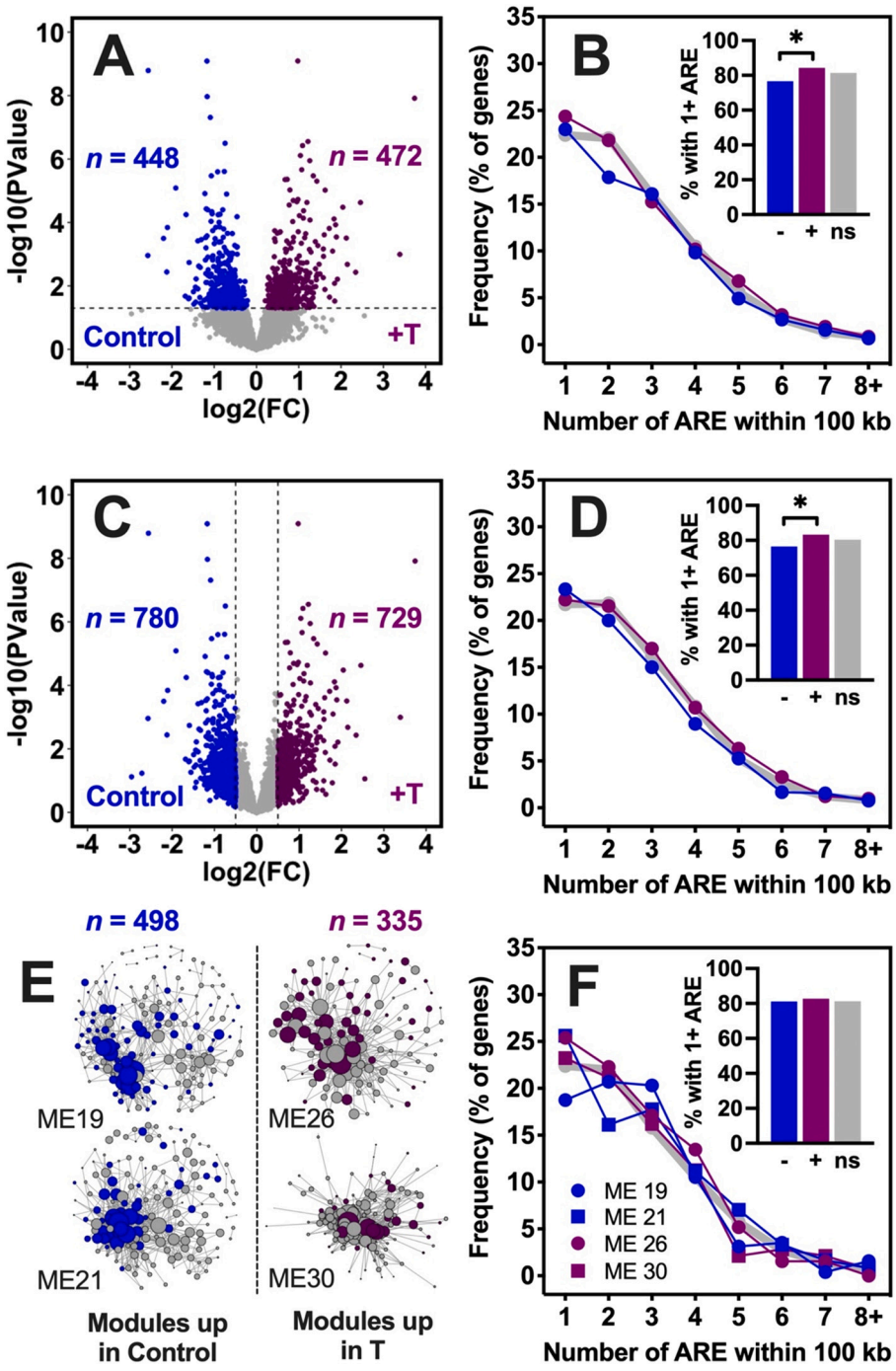


Fig. 6. Transcriptome-wide analysis of T-mediated gene expression in *A. sagrei* liver and the proximity of putative androgen response elements (ARE) in the genome of *A. carolinensis*. (A) Volcano plot differentiating gene expression (2 months post-treatment) as a function of the fold change in $\log_2$ expression ($\log_2$ FC) between animals receiving T implant or control. Genes that are differentially expressed ($P < 0.05$ without correction for false discovery) are indicated in blue (higher in control) or purple (higher in T). (B) Frequency distribution of the number of putative AREs on either DNA strand within 100 kb for three classes of genes (blue = down-regulated by T, purple = up-regulated by T, gray = not regulated by T) as identified in panel A. Inset panels show the cumulative percentage of genes in each category that are proximate to at least 1 putative ARE. Asterisks indicate significant differences between up- and down-regulated genes. (C) The same volcano plot as in panel A, with differential expression classified as $\log_2$ FC < -0.5 (higher in control) or $\log_2$ FC > 0.5 (higher in T). (D) Frequency distributions analogous to panel B, but based on gene classifications from panel C. (E) Gene co-expression modules up-regulated in the control group (left) and up-regulated in T group (right; see Fig. 5 and Table S2). (F) Frequency distributions analogous to panels B and D, but based on gene membership in the T-responsive modules from panel E relative to all other expressed genes. In each comparison, frequency distributions for ARE proximity are very similar across all gene expression categories, although insets reveal that genes up-regulated by T are slightly more likely to be proximate to at least 1 ARE, relative to genes that are more highly expressed in controls.

may often correlate with the regulation of target genes (Frankl-Vilches et al., 2015; Fuxjager et al., 2016). We therefore tested whether genes that are up- or down-regulated in response to T differ in the presence and number of proximate AREs relative to one another and to genes that are unresponsive to T. We defined responsiveness to T based on (1) an uncorrected $P < 0.05$ for the effect of T treatment (Fig. 6A), or (2) an absolute value of $\log_2$ fold-change >0.5 when comparing T and control groups (Fig. 6C). Although genes that are up-regulated by T are slightly more likely to have at least one proximate ARE relative to genes that are expressed more highly in controls (inset panels in Fig. 6B, D, S4), frequency distributions of the number of proximate AREs are similar regardless of whether genes are up-regulated by T, down-regulated by T, or unbiased relative to T (Fig. 6B, D, S4). Likewise, the distribution of proximate AREs is similar for genes within modules that are collectively up- or-down regulated by T (i.e., modules 19, 21, 26, and 30; Fig. 5C-F; Table S4) when compared to the distribution of AREs for expressed genes in other modules (Figs. 6E-F, S4G—I).

Therefore, patterns of hepatic gene expression in response to chronically elevated T in *A. sagrei* are only weakly and inconsistently associated with the presence of proximate AREs predicted from in silico analysis of the *A. carolinensis* genome. Previous studies of gene expression in the songbird brain (Frankl-Vilches et al., 2015) or flight muscle (Fuxjager et al., 2016) have found more robust signatures of ARE enrichment for T-responsive genes using in silico approaches. One obvious caveat to our approach is that the genome of a congener may not accurately represent the regulatory features of our focal species, which is particularly relevant given our emphasis on the evolutionary lability of AREs. Indeed, comparisons between more distantly related genomes in

other taxa (e.g., comparisons of canary or manakin to zebra finch genomes) suggest frequent gains and losses of AREs (Frankl-Vilches et al., 2015; Fuxjager et al., 2016). Another difference is that we considered all AREs within several windows of proximity to transcriptional start sites (Figs. 6; S4), but we did not restrict our analyses to AREs within putative promoter regions (Frankl-Vilches et al., 2015; Fuxjager et al., 2016), which should be captured primarily within our 5 kb proximity window (see Fig. S4). It is also likely that many effects of T on gene expression reflect *trans* regulation. In particular, the differences in gene expression that we characterized several months post-treatment are likely due in part to long-term reorganization of the liver transcriptome in the presence of chronically elevated T, rather than (or in addition to) direct ARE-mediated effects of T on target genes. Discerning among these possibilities is beyond the scope of this study, but it serves to illustrate some of the many challenges inherent in linking readouts of hormonally mediated gene expression with regulatory features in the genome.

The in silico identification of putative AREs can potentially reveal broad patterns when averaging across entire classes of genes (e.g., T-responsive versus non-responsive genes; Fig. 6) or when comparing proportions of genes with proximate AREs across species (Frankl-Vilches et al., 2015; Fuxjager et al., 2016). However, our results suggest that genome-wide in silico methods are poorly suited for more precise characterizations on gene-by-gene basis, for which direct evidence of AR binding to DNA in the tissue of interest may often be necessary. As such, in silico methods may lack the level of precision needed to address ARE evolution as envisioned, for example, in our Fig. 1D. In silico methods for ARE identification are refined on the basis of chromatin immunoprecipitation followed by sequencing (ChIP-seq) that identifies physical interactions between AR and DNA (Wilson et al., 2016). Comparative studies have leveraged ChIP-seq to document widespread evolutionary changes in the binding sites for a variety of other non-AR transcription factors (Schmidt et al., 2010; Villar et al., 2015; Villar et al., 2014). The evolutionary inferences from these studies are broadly similar to those from several in silico comparisons of AREs across avian genomes (Frankl-Vilches et al., 2015; Fuxjager et al., 2016), in the sense that they collectively support the view that transcription-factor binding sites evolve rapidly across species. One particularly promising future avenue for characterizing evolutionary change at the hormone-genome interface will be to compare divergent species or populations with respect to direct readouts of AR/ARE interactions at the hormone-genome interface, particularly when combined with comparative studies of chromatin accessibility and tissue-wide gene expression.

6. Synthesis and future directions

Our central message is that the field of evolutionary endocrinology will benefit from a perspective that goes beyond the evolution of hormonal signals to simultaneously explore the evolution of hormone-phenotype couplings and hormone-genome interactions. This is certainly not a new message (e.g., Bergeon Burns et al., 2014; Cox, 2020; Fuxjager and Schuppe, 2018; Hau, 2007; Husak and Lovern, 2014; Lipshutz et al., 2019), but we hope that our presentation of general ideas and organizing principles will aid colleagues in connecting this perspective to their own work. Our classification of general mechanisms for hormone-phenotype couplings (Fig. 1) is vastly oversimplified and leaves out many important details (and some potentially important mechanisms, such as epigenetic modification and the evolution of hormone receptors themselves). Even with these simplifications, it illustrates the multi-layered complexity of hormone-phenotype couplings and serves as a reminder that most comparative work to date has focused on tissue sensitivity and receptor expression (Fig. 1C). Downstream features of the hormone-genome interface (Fig. 1D-E) represent an exciting new frontier for evolutionary endocrinology in the age of comparative genomics.

Through re-analysis of a recent study, we found that a sexual signal used in behavior displays exhibits additive genetic variance (Fig. 3) and covariance (Fig. 4) in its responsiveness to a standardized hormonal signal, which can also be conceptualized as additive genetic variance in the architecture of hormonal pleiotropy. To our knowledge, this is the first such demonstration of this phenomenon. One limitation of our approach (and of statistical quantitative genetics in general) is that it leaves the mechanistic basis of this genetic variance unresolved. Although similar approaches could address this limitation by characterizing genetic variance in receptor density or expression of key target genes (e.g., via quantitative PCR), is unlikely that experiments of similar scope (i.e., requiring hormone manipulations in hundreds to thousands of individuals with known genetic relationships) will be feasible for many systems. Nonetheless, our findings confirm that hormone-phenotype couplings are heritable and emphasize the evolutionary significance of individual variation in phenotypic responses to hormone signals.

We also show that weighted gene co-expression network analysis can help visualize and quantify patterns of T-mediated gene expression that are not readily apparent from differential expression analysis of individual genes. On the other hand, these modules do not necessarily map to biological processes that help us interpret the functional significance of T-mediated changes, and the relationship between statistically defined modules and mechanistic pathways is unclear. Likewise, we do not see a clear signature of *cis* regulation via androgen response elements when combining our gene expression data with in silico analysis of a congener genome. While there are admittedly many ways in which we could extend and improve our analysis beyond what we present here as an illustrative example, it may also generally be true that in silico methods are best suited for broad "proof-of-concept" inferences that quantify overall differences in ARE distribution or number across broad classes of genes or between different genomes, with limited utility for pinpointing exact locations of evolutionary change. Related approaches that focus on direct interactions between hormone receptors and DNA (e.g., ChIP-seq) hold great promise for comparative evolutionary endocrinology. Regardless of the approaches used, comparative studies of gene expression and hormone-genome interactions are needed to balance our expanding understanding of the evolution of circulating hormone levels with a complementary understanding of the evolution of hormone-phenotype couplings.

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CRediT authorship contribution statement

RMC conceived of the main outline for the paper and supervised the project; TNW, CDR, and RMC generated the quantitative genetic dataset; TNW conducted the quantitative genetic analyses; CLC generated the RNA-seq data; MDH and CDR conducted the gene expression and in silico genome analyses; TNW, MDH, and RMC created the figures and tables; RMC prepared the initial manuscript draft with assistance from TNW, MDH, and CDR; all authors contributed to the final version of the manuscript.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.yhbeh.2022.105216.

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