Molecular and Cellular Endocrinology 502 (2020) 110668

Contents lists available at ScienceDirect

Molecular and Cellular Endocrinology

journal homepage: www.elsevier.com/locate/mce

Sex steroids as mediators of phenotypic integration, genetic correlations, and evolutionary transitions

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ARTICLE INFO

Keywords:
Evolutionary endocrinology
Genetic correlation
Hormonal pleiotropy
Phenotypic integration
Regulatory evolution
Transcriptome

ABSTRACT

In recent decades, endocrinologists have increasingly adopted evolutionary methods and perspectives to characterize the evolution of the vertebrate endocrine system and leverage it as a model for developing and testing evolutionary theories. This review summarizes recent research on sex steroids (androgens and estrogens) to illustrate three ways in which a detailed understanding of the molecular and cellular architecture of hormonally mediated gene expression can enhance our understanding of general evolutionary principles. By virtue of their massively pleiotropic effects on the expression of genes and phenotypes, sex steroids and their receptors can (1) structure the patterns of phenotypic variance and covariance that are available to natural selection, (2) alter the underlying genetic correlations that determine a population's evolutionary response to selection, and (3) facilitate evolutionary transitions in fitness-related phenotypes via subtle regulatory shifts in underlying tissues and genes. These principles are illustrated by the author's research on testosterone and sexual dimorphism in lizards, and by recent examples drawn from other vertebrate systems. Mechanistically, these examples call attention to the importance of evolutionary changes in (1) androgen- and estrogen-mediated gene expression, (2) androgen and estrogen receptor expression, and (3) the distribution of androgen and estrogen response elements in target genes throughout the genome. A central theme to emerge from this review is that the rapidly increasing availability of genomic and transcriptomic data from non-model organisms places evolutionary endocrinologist in an excellent position to address the hormonal regulation of the key evolutionary interface between genes and phenotypes.

1. Introduction

Over the past several decades, the field of evolutionary endocrinology has emerged as a synthesis between evolutionary genetics and comparative endocrinology (Cox et al., 2016a; Nepomnaschy et al., 2009; Zera et al., 2007). Consequently, it is now routine for comparative studies of hormonal phenotypes to adopt formal phylogenetic methods (Goymann et al., 2018; Husak and Lovern, 2014; Johnson et al., 2018a; Vitousek et al., 2018). Likewise, comparative endocrinology has leveraged the availability of genetic and genomic data from non-model vertebrates to characterize the molecular evolution of hormone receptors (Eick and Thornton, 2011; Filowitz et al., 2018; Frankl-Vilches and Gahr, 2018; Sumiya et al., 2015), genes in their signaling pathways (Lorin et al., 2015), and recognition motifs in their target genes (Frankl-Vilches et al., 2015; Fuxjager and Schuppe, 2018). In wild populations, phenotypic selection analyses have been adapted to measure natural selection on both baseline hormone levels and those induced by experimental stress or hormone manipulation (Bonier and Cox, 2020, Bonier and Martin, 2016; Cox et al., 2016b; John-Alder et al., 2009; McGlothlin et al., 2010; Patterson et al., 2014). To assess the evolutionary consequences of such selection, quantitative genetic studies of heritability and genetic correlations are also being used to characterize the genetic architecture of endocrine phenotypes and thereby predict how they will respond to selection (Cox et al., 2016b; Iserbyt et al., 2015; Pavitt et al., 2014; Stedman et al., 2017).

The approaches described above represent the successful application of foundational evolutionary techniques to explore the evolution of the endocrine system itself. However, there are also many ways in which the study of endocrinology can inform broader theoretical issues in evolutionary biology. For example, the divergence of androgen, progestogen, corticosteroid, and mineralocorticoid receptors following gene duplication of an ancestral estrogen receptor has been used to explore evolutionary concepts ranging from exaptation and irreducible complexity (Bridgham et al., 2006; Thornton, 2001) to the importance of historical contingency and resultant predictability and reversibility of evolution (Bridgham et al., 2009; Harms and Thornton, 2014; Starr et al., 2017). Likewise, the recognition that most major axes of the vertebrate endocrine system are both evolutionarily conserved and

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https://doi.org/10.1016/j.mce.2019.110668
Received 17 March 2019; Received in revised form 19 November 2019; Accepted 26 November 2019
Available online 09 December 2019
0303-7207/

responsible for pleiotropically regulating numerous genes and phenotypes has stimulated considerable discussion about the extent to which this type of regulatory architecture acts as a source of evolutionary potential versus constraint (Fuxjager and Schuppe, 2018; Hau, 2007; Ketterson and Nolan, 1999; Lema, 2014; McGlothlin and Ketterson, 2008). In essence, this is an endocrine-centric version of the much broader question of whether and how evolutionary trajectories are influenced by the functional mapping of genes to phenotypes (Pigliucci, 2010; Wagner and Zhang, 2011).

The goal of this paper is to call attention to several ways in which the study of hormones in general, and sex steroids in particular, can inform broader questions in evolutionary biology. For simplicity, this review focuses on androgens and estrogens, though many of the underlying principles are generalizable other hormones, particularly those that exert genomic effects by altering transcription of target genes. Although sex steroids can produce rapid (non-genomic) cellular responses without altering gene expression (Lösel and Wehling, 2003), their ability to pleiotropically regulate the transcription of hundreds to thousands of genes is key to their evolutionary significance, so this review begins with a brief discussion of the mechanisms by which sex steroids link genes to phenotypes via transcription. The coordinated regulation of multiple phenotypes by androgenic and estrogenic pathways is predicted to structure patterns of phenotypic integration (Fig. 1), thereby shaping the trait combinations that are available to selection. Although the underlying principle of hormonal pleiotropy (Fig. 1A) is widely recognized, it has yet to be rigorously incorporated into a quantitative genetic framework centered on phenotypic variance and covariance. Moreover, because sex steroids regulate gene expression, they also have the potential to shape the underlying patterns of genetic covariance (Fig. 1C) that determine how populations evolve in response to natural and sexual selection on correlated traits. For example, sex steroids can orchestrate the developmental breakdown of genetic correlations between males and females, thereby facilitating sex-specific phenotypic evolution despite the constraint of a shared genome.

Shifting from a quantitative genetics (microevolutionary) framework to a phylogenetic (macroevolutionary) perspective, several recent examples are used to illustrate how evolutionary transitions in sexually selected phenotypes have been linked to the molecular and cellular coupling (or decoupling) of phenotypes to (or from) androgenic and estrogenic signaling. These couplings can occur at the tissue level, through changes in receptor density, cofactor availability, and resultant sensitivity to sex steroids, or at the gene level, through the gain or loss of recognition motifs in regulatory regions. In extreme cases, evolutionary reversals in sexual dimorphism may be achieved by reversing the effects of a hormone on a given phenotype, as illustrated by the

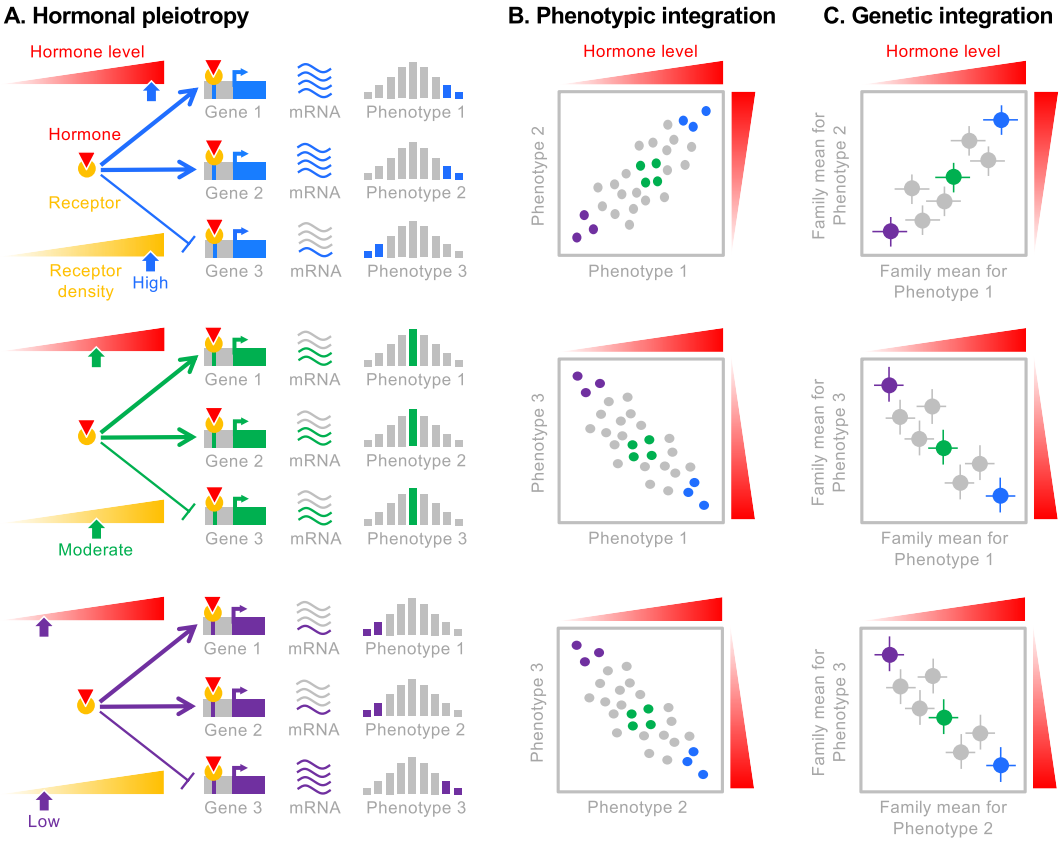

Fig. 1. (A) **Hormonal pleiotropy** occurs when a single hormone (red triangle) and its receptor (yellow circle) bind response elements in the promoter regions of different genes to simultaneously enhance (arrows) or repress (blunted lines) the expression of mRNA and the distribution of multiple downstream phenotypes. Different patterns of expression for mRNA and downstream phenotypes are shown for situations corresponding to high (blue), moderate (green), and low (purple) levels of circulating hormone and/or receptor expression. For simplicity, increased expression of a gene is presumed to directly increase the expression of its associated phenotype. **(B) Phenotypic integration** can occur as a consequence of variation in circulating hormone levels (or variation in receptor density and other factors involved in mediating genomic effects of hormones) and manifests as positive or negative covariances between phenotypes across individuals (small circles). This phenotypic (co)variance can be due to a combination of genetic factors, environmental factors, and their interactions, each of which can alter levels of hormones, receptors, and gene expression. **(C) Genetic integration** occurs when this phenotypic (co)variance has a heritable genetic basis, as illustrated in a situation where those individuals with similar hormone/receptor levels in panel A and similar phenotypes in panel B are related to one another (e.g., siblings; large symbols represent family means, error bars represent variance within families). If selection directly favors high values for Phenotype 1 (blue individuals), it will also indirectly favor high values for Phenotype 2 and low values for Phenotype 3 due to phenotypic integration. If phenotypic integration is underlain by genetic integration, Phenotypes 2 and 3 will evolve in response to selection on Phenotype 1 (and vice versa).

"bipotential" nature of testosterone as both a promoter and an inhibitor of growth across reptiles. The cellular and molecular mechanisms that facilitate such evolutionary reversals are not yet known, but represent a promising path for future research. Collectively, these topics illustrate how the increasing accessibility of genomic and transcriptomic data for non-model organisms can be leveraged to address emerging hypotheses about the roles of sex steroids in shaping phenotypic integration, genetic correlations, and evolutionary transitions.

2. Sex steroids as links between genes and phenotypes

Sex steroids (androgens, estrogens, progestogens) are produced and secreted into circulation primarily, but not exclusively, by the sexually differentiated gonads (ovaries, testes) in response to circulating gonadotropins (lutenizing hormone, LH; follicle stimulating hormone, FSH) that are themselves released by the anterior pituitary in response to gonadotropin-releasing hormone (GnRH) from the hypothalamus. Collectively, these tissues and hormones comprise the hypothalamic-pituitary-gonadal (HPG) axis, which coordinates development, sexual maturation, reproduction, and behavior, among other things. Although the gonads represent primary point at which HPG function diverges between the sexes, the hypothalamus and especially the pituitary also exhibit sexually differentiated transcriptomes (MacManes et al., 2017). Once sex steroids are in circulation, sex hormone-binding globulins (SHBG) limit the availability of biologically active (unbound) hormone (Laurent et al., 2016). Upon delivery to target tissues, unbound androgens and estrogens exert their phenotypic effects by two primary modes of action: genomic and non-genomic. Non-genomic effects can occur through several signaling pathways that share the common property of a rapid (< 10 min) cellular response that does not involve transcriptional regulation (Foradori et al., 2008; Lösel and Wehling, 2003). Because transcriptional regulation is key to the evolutionary significance of sex steroids as links between genes and phenotypes, this review focuses on the genomic effects of sex steroids.

Genomic effects occur via the diffusion of sex steroids across the plasma membranes of target cells, where they bind nuclear receptors (androgen receptor, AR; estrogen receptors, ERα, ERβ) in the cytosol. Binding is often preceded by local conversion of the steroid to another biologically active metabolite, as in the case of testosterone (T) conversion to the more potent androgen 5α-dihydrotestosterone (DHT) by 5α-reductase, or to the estrogen estradiol (E_2) by aromatase. Binding by the steroid ligand initiates a conformational change in the nuclear receptor that causes dissociation of heat shock proteins, translocation of the receptor into the cell nucleus, and dimerization to form an active receptor that recruits necessary cofactors and polymerases to initiate transcription of target genes (Fuxjager and Schuppe, 2018; Gobinet et al., 2002; Gruber et al., 2004). The DNA-binding domain of the receptor contains two binding sites that recognize short sequences of DNA in the promoter regions of target genes. These short DNA sequences (androgen response elements, AREs; estrogen response elements, EREs) typically consist of two inverted repeats separated by three variable (n) nucleotides (e.g., ARE consensus motif: 5′-AGAACAnnnTGTTCT-3'; ERE consensus motif: 5′-AGGTCAnnnTGACCT-3′) (Filho et al., 2019; Starr et al., 2017). Although base identity is highly conserved at some positions in these inverted repeats, other positions are more variable, such that alternative recognition motifs are also bound by ARs and ERs (Gruber et al., 2004; Mason et al., 2010; Nelson et al., 1999; Wilson et al., 2016). Direct repeats (e.g., 5′-AGAACAnnnAGAACA-3′) can provide additional, "selective" response elements for ARs that reduce binding by related glucocorticoid (GC) and mineralocorticoid (MC) receptors (Denayer et al., 2010). The location of a response element within the promoter can determine whether it represses or enhances expression, and the number of additional response elements present in the promoter to cooperatively enhance DNA binding can influence the overall level of expression (Gruber et al., 2004; Reid et al., 2001). Whether gene expression is repressed or enhanced following receptor binding to response elements also depends upon the various cofactors that are recruited to the transcription complex (Shang et al., 2002), which in turn can depend upon the identity of the ligand bound to the receptor, as in the case of the differential regulation induced by the ER depending upon whether it is bound by estrogen or the antagonist tamoxifen (Shang et al., 2000).

Genomic regions that are bound by AR and ER can be identified by chromatin immunoprecipitation followed by microarray or RNA sequencing (Cheung and Kraus, 2010). Estimates of the number of unique binding regions for ARs and ERs throughout the genomes of mice and humans range from several thousand in studies with predetermined probe sets or tiling arrays (Carroll et al., 2006; Gao et al., 2008; Lin et al., 2007; Mason et al., 2010), to tens of thousands in transcriptome-wide analyses (Hewitt et al., 2012; Hu et al., 2010; Welboren et al., 2009; Wyce et al., 2010; Yao et al., 2017). Promoter regions located immediately upstream from annotated genes are typically enriched for AR and ER binding sites on a per-nucleotide basis, but the majority of AR and ER binding sites actually occur in introns and intergenic regions (Gao et al., 2008; Hu et al., 2010; Wyce et al., 2010; Yao et al., 2017). Estimates of the total number of genes that are differentially expressed in response to androgen or estrogen treatment range from several hundred to several thousand genes for any given tissue, across a range of species and sample sizes (Bramble et al., 2016; Cox et al., 2017b; Frankl-Vilches et al., 2015; Fuxjager et al., 2016; Peterson et al., 2013, 2014; Snyder et al., 2009; van Nas et al., 2009; Xu et al., 2012; Zheng et al., 2013). Estimates of the percentage of such differentially expressed genes with proximate AR or ER binding sites range from 51 to 71% across several tissues and studies, indicating a combination of *cis*- and *trans*-regulation by sex steroids (Lin et al., 2007, 2009; Welboren et al., 2009).

To appreciate the evolutionary significance of sex steroids as links between genes and phenotypes, it helps to distill the regulatory complexity described above into a simplified model with several key features. First, sex steroids are produced and secreted such that their circulating titers provide important contextual signals (e.g., sex, age, season, reproductive status) that are broadcast throughout the body. Second, responsiveness to these global signals can be altered at the local cellular level to achieve tissue-specificity and further refine specificity with respect to sex, age, or reproductive status. Third, vertebrate genomes contain tens of thousands of unique recognition sites at which sex steroids can interact with DNA to coordinate the expression of hundreds to thousands of genes. Collectively, this means that different signals (e.g., androgens or estrogens) can produce dramatically different phenotypes (e.g., male and female) from the same underlying genes. Moreover, variance in both signal (e.g., circulating hormone titers) and responsiveness (e.g., levels of receptors, binding globulins, and cofactors for transcription) is predicted to lead not only to variance in individual phenotypes, but also to different patterns of covariance between these phenotypes. These phenotypic variances and covariances, and their underlying genetic variances and covariances, are central to evolutionary quantitative genetics.

3. Sex steroids as mediators of phenotypic integration

Scaling up to the organismal level, the genome-wide coordination of transcription that is achieved by AR and ER binding results in hormonal pleiotropy (Fig. 1A), the regulation of multiple phenotypes by a single hormone (Cox et al., 2009b; Hau, 2007; Lema, 2014; McGlothlin and Ketterson, 2008). Hormonal pleiotropy is analogous to the "one-to-many" pleiotropic mapping of genes to phenotypes (Wagner and Zhang, 2011), albeit with a hormone ligand and its receptor substituting for a gene in the literal sense. Likewise, multiple hormones can interact to influence the expression of a single gene (e.g., one promoter region may recognize AR, ER, and related steroid receptors due to separate recognition elements or lack of receptor specificity) or a single organismal phenotype. Similar phenomena have occasionally been referred to as

intermolecular epistasis (Anderson et al., 2015) and physiological epistasis (Lancaster and Sinervo, 2011; Sinervo and Calsbeek, 2003; Sinervo et al., 2008), but "hormonal epistasis" has apparently never caught on as a counterpart to hormonal pleiotropy. As with genetic pleiotropy, hormonal pleiotropy can be viewed as both an adaptation for coordinating the expression of phenotypes that interactively influence fitness, and as a potential constraint on further adaptation (Adkins-Regan, 2008; Dantzer and Swanson, 2017; Hau, 2007; Ketterson and Nolan, 1999; Lema, 2014; McGlothlin and Ketterson, 2008). This latter view of evolutionary constraint is based on the reasoning that any fitness benefits due to changes in an endocrine pathway with respect to one function will tend to be offset by the disruption of other functions. As with classic genetic pleiotropy (Paaby and Rockman, 2013; Stern, 2000), the relevance of such constraint may depend largely upon one's definition of hormonal pleiotropy (Lema, 2014).

Hormonal pleiotropy leads to phenotypic integration (Fig. 1B), which can be conceptually and mathematically defined in a number of ways (Armbruster et al., 2014), but typically involves patterns of correlation between phenotypes (Conner et al., 2014; Murren, 2012). In evolutionary quantitative genetics, these patterns are formalized as measures of phenotypic variance, which describes the variation in a trait that is available to natural selection, and phenotypic covariance, which describes the trait combinations that are available to selection. If the covariance between two traits is high (Fig. 1B), selection cannot act on one trait without also influencing the other (Lande and Arnold, 1983). From an endocrine perspective, phenotypic variance within a population could potentially arise from (1) variance among individuals in any aspect of the HPG axis that results in differential production, secretion, and bioavailability androgens and estrogens (i.e., hormonal signal); (2) variance among individuals in AR and ER densities, reductase and aromatase levels, and availability of transcription cofactors in target tissues (i.e., tissue-specific responsiveness), and (3) variance among individuals in ARE and ERE locations and sequence motifs throughout the genome (i.e., gene-specific responsiveness). Hormonal pleiotropy has served as an important organizing concept in evolutionary endocrinology (Finch and Rose, 1995; Ketterson and Nolan, 1999; Williams, 2012), but it is usually invoked in the context of a single hormone manipulation altering the means of two or more response variables (e.g., Cox et al., 2009b), without explicit consideration of corresponding effects on the phenotypic variances and covariances that form the basis of statistical quantitative genetics and multivariate analyses of selection. Nonetheless, a simple manipulation of hormone levels can serve as an illustrative proof of concept.

In the brown anole lizard (*Anolis sagrei*), treatment of juvenile females with exogenous T causes phenotypic masculinization by increasing growth rate and altering the size and coloration of the dewlap, a sexual ornament (Fig. 2A and B) (Cox et al., 2015). Phenotypic variance in growth rate and dewlap morphology is significantly higher in T-supplemented females than in control females with uniformly low plasma T levels, and dramatically higher when considering both treatment groups together (Fig. 2C). This latter point is somewhat trivial, but it can be viewed as an empirical proof of concept that increasing the phenotypic variance in circulating T increases phenotypic variance in other T-mediated traits. Likewise, traits that are independently influenced by T exhibit increased phenotypic covariance (correlation) across treatment groups, relative to natural levels of covariance observed within the control group (Fig. 2D). Again, this serves as an empirical proof of concept that, with increased variance in circulating T, other traits influenced by T will tend to exhibit increased phenotypic covariance (Cox et al., 2016b).

Because most endocrine manipulations involve small sample sizes (e.g., 5–15 individuals per group), this empirical proof of concept is typically the limit of what we can causally infer about the role of hormonal pleiotropy in shaping phenotypic integration. However, larger sample sizes (e.g., 200 individuals per group) reveal that patterns of phenotypic correlation within T-supplemented females can differ substantially from natural patterns in control females (Fig. 2D). For example, a strong negative correlation between dewlap area and brightness is induced by T in females ($r = -0.40$; $P < 0.001$; $n = 201$; Fig. 2D), which differs dramatically from the weak positive correlation observed under naturally low T levels ($r = 0.10$; $P = 0.17$; $n = 190$; Fig. 2D), but is similar to negative correlations found within T-supplemented males ($r = -0.22$; $P < 0.001$; $n = 203$) and intact control males ($r = -0.27$; $P = 0.002$; $n = 191$) (T. Wittman, C.D. Robinson, R.M. Cox, unpublished data). It is also reminiscent of the weak negative genetic correlation ($r_g = -0.21$) between dewlap area and brightness in intact adult males (Cox et al., 2017a). This creation of phenotypic covariance by T implies that females harbor underlying variance in responsiveness that is only evident when T is present, and which could potentially occur via any of the tissue- or gene-specific mechanisms discussed above. As described in detail elsewhere, these patterns of hormonally mediated phenotypic integration can be expressed as phenotypic variance-covariance matrices and synthesized into standard quantitative genetics approaches (Cox et al., 2016b). However, as illustrated above, integrating hormonal pleiotropy within the statistical framework of evolutionary quantitative genetics will often require relatively large sample sizes for accurate estimation of variances and covariances (e.g., Fig. 2D), which may explain why most attempts at synthesis have been largely theoretical (Cox et al., 2016b; Dantzer and Swanson, 2017; Ketterson et al., 2009, but see McGlothlin et al., 2010).

How might a more detailed understanding of molecular and cellular endocrinology improve our understanding of phenotypic integration? As a first step, characterizing hormonal pleiotropy and phenotypic integration at the level of the "primary phenotype" of gene expression could help to address a number of issues. For example, weighted gene coexpression network analyses (WGCNA, Langfelder and Horvath, 2008) could be used to identify modules of genes with highly correlated expression and then derive "eigengenes" (first principle components) for each module. This approach has been used to characterize sex- and tissue-specific expression modules in mice (van Nas et al., 2009), and can be extended to identify hormonally mediated modules. In the context of phenotypic integration, the "eigengene" PC1 scores of individuals could stand in for phenotypic trait values to (1) determine which modules (phenotypes) are responsive to hormones (analogous to Fig. 2A), (2) assess variance and covariance in module expression across individuals (analogous to Fig. 2C–D), (3) determine how hormones influence the strength of connectivity within modules (a nested level of phenotypic integration), and (4) test whether particular modules are unique to a particular hormonal context. Potential advantages of this transcriptomic approach over traditional phenotypic (co)variance analyses include the relative abundance of expression phenotypes, the potential to select phenotypes in an unbiased fashion, and the ability to directly link genes and phenotypes by focusing on mRNA.

Comparison of hormonally mediated gene expression across sexes or tissues could also be used to assess the lability (evolutionary potential) or conservation (evolutionary constraint) of hormonal pleiotropy when allowing for natural sex- and tissue-specific regulation. This could be viewed as an indirect test of the extent to which the genomic distribution of AREs and EREs, which is constant across sexes (for autosomes) and tissues, predisposes a species to a fixed pattern of hormonal pleiotropy. Such studies might ideally adopt a network approach (van Nas et al., 2009), though more straightforward comparisons of differentially expressed genes have also revealed high degree of sex- and tissue-specificity in transcriptomic responses to sex steroids (Bramble et al., 2016; Peterson et al., 2013, 2014; Snyder et al., 2009; Xu et al., 2012; Zheng et al., 2013). However, most have not been interpreted in the context of hormonal pleiotropy (but see Peterson et al., 2014). Analogous comparisons of pleiotropic networks or expression patterns across species (within sexes and tissues) can test the extent to which hormonally mediated expression profiles are evolutionarily conserved versus labile.

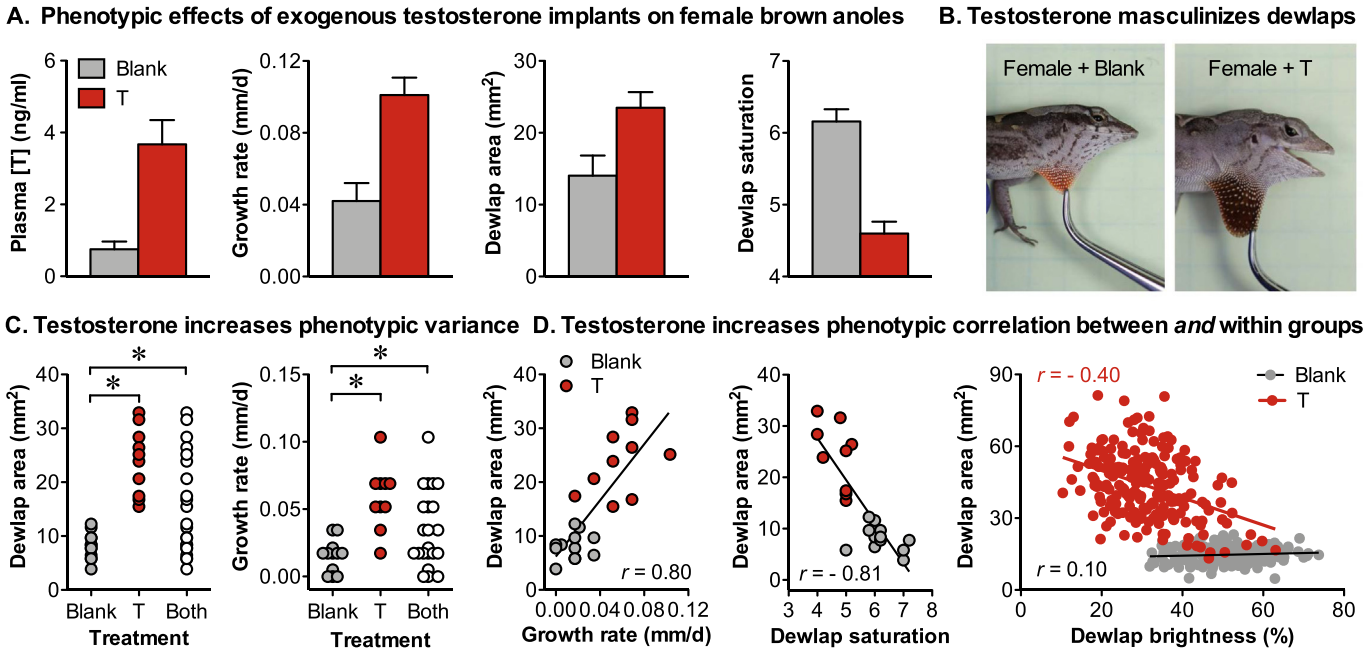

Fig. 2. (**A**) Treatment of juvenile female brown anoles with exogenous testosterone (T) elevates plasma [T], stimulates growth, and masculinizes the dewlap, as also shown qualitatively in (**B**). (**C**) The T group exhibits significantly greater phenotypic variance, relative to the blank control group, in traits such as dewlap area and growth rate. Phenotypic variance is substantially greater across both treatment groups combined. Asterisks (*) indicate significant ($P < 0.05$) differences in variance relative to the control group based on F tests. (**D**) Enhanced phenotypic variance, coupled with directional effects of T on phenotypic means, creates strong phenotypic correlations (r) between traits when assessed across treatment groups (left panels). Given sufficient sample sizes (right panel), T can be shown to increase phenotypic correlation within the treatment group relative to the control group, implying underlying variance in responsiveness to T. Adapted from Cox et al. (2016b) in Integrative and Comparative Biology.

4. Sex steroids as mediators of genetic correlations

Although hormonal pleiotropy is widely regarded as a source of phenotypic integration, the ability of AR and ER to interact directly with DNA as transcription factors gives sex steroids a less widely appreciated ability to simultaneously shape patterns of genetic integration (Cox et al., 2016b). Genetic integration (Fig. 1C) is analogous to phenotypic integration (Fig. 1B), but with genetic variance (standardized as heritability) and genetic covariance (standardized as genetic correlation) taking the place of phenotypic variance and covariance. These parameters are typically estimated from phenotypic measurements in combination with information on genetic relatedness of individuals (e.g., from a controlled breeding design or pedigreed population, reviewed by Cox et al., 2016b for steroid hormones). Genetic variance determines the extent to which selection on phenotypic variance will result in evolutionary change across generations. Likewise, genetic covariance determines the extent to which selection acting on one trait will lead to a correlated evolutionary response by other traits (Lande, 1980; Lande and Arnold, 1983). Simple models in which the separate effects of a hormone on each of two phenotypes substitute for a traditional genetic correlation between two phenotypes illustrate the potential for hormonal pleiotropy to shape genetic covariance and alter evolutionary trajectories (Dantzer and Swanson, 2017). Likewise, more complex models incorporating evolving peptide hormones and hormone-receptor binding dynamics illustrate how the evolution of hormonal pleiotropy itself can structure phenotypic covariance and shape life-history tradeoffs (Bourg et al., 2019).

Because sex steroids circulate throughout the body to regulate hundreds of genes and phenotypes, one might also view the hormonal milieu of an individual as akin to the environment in which its genes are expressed (Cox et al., 2016b). Different environments often lead to different patterns of genetic variance and covariance for a variety of phenotypes (reviewed by Wood and Brodie, 2015). By analogy, different endocrine environments (e.g., high androgen levels in a "male" environment versus high estrogen levels in a "female" environment), have the potential to produce different (e.g., sex-specific) patterns of genetic correlation for hormonally mediated traits. One way to test this hypothesis would be to split full siblings from a controlled breeding experiment into different hormone treatments and then compare patterns of genetic correlation for various hormonally mediated phenotypes across the hormone treatments. As noted above, studies of phenotypic variance and covariance require large sample sizes, and this is even more of a concern for studies of genetic variance and covariance.

An illustrative example of how sex steroids can structure genetic correlations comes from the inferred role of T in reducing genetic correlations between the sexes (Cox et al., 2017b). A between-sex genetic correlation (r_{mf}) quantifies the extent to which heritable variation in a phenotype is correlated between males and females (Cox et al., 2017a). From an evolutionary perspective, a high value of r_{mf} represents a constraint because it indicates that selection on the phenotype in one sex will result in a correlated evolutionary response in the opposite sex, thereby constraining the independent evolution of the sexes (Lande, 1980, 1987). The evolution of sexual dimorphism is therefore predicted to proceed through evolutionary reductions in r_{mf} (Delph et al., 2011; Fairbairn and Roff, 2006), but the physiological and genetic mechanisms that facilitate such breakdown of r_{mf} are not well understood (Cox et al., 2017b). In brown anole lizards, sex differences in size are absent at hatching but develop rapidly as males and females mature (Fig. 3A). Before the sexes diverge in size, r_{mf} is high, but as sexual dimorphism emerges during the transition from juvenile to subadult life stages, r_{mf} declines rapidly (Fig. 3B). This breakdown in r_{mf} coincides with maturational increases in circulating T levels and with the expression of other androgen-mediated phenotypes (e.g., enlarged dewlaps) in males, thus implicating T.

The developmental breakdown of r_{mf} for body size also coincides with a pronounced increase in the number of significantly sex-biased genes in the liver transcriptome, and in the average magnitude of sex-biased expression, particularly for genes in key growth-regulatory

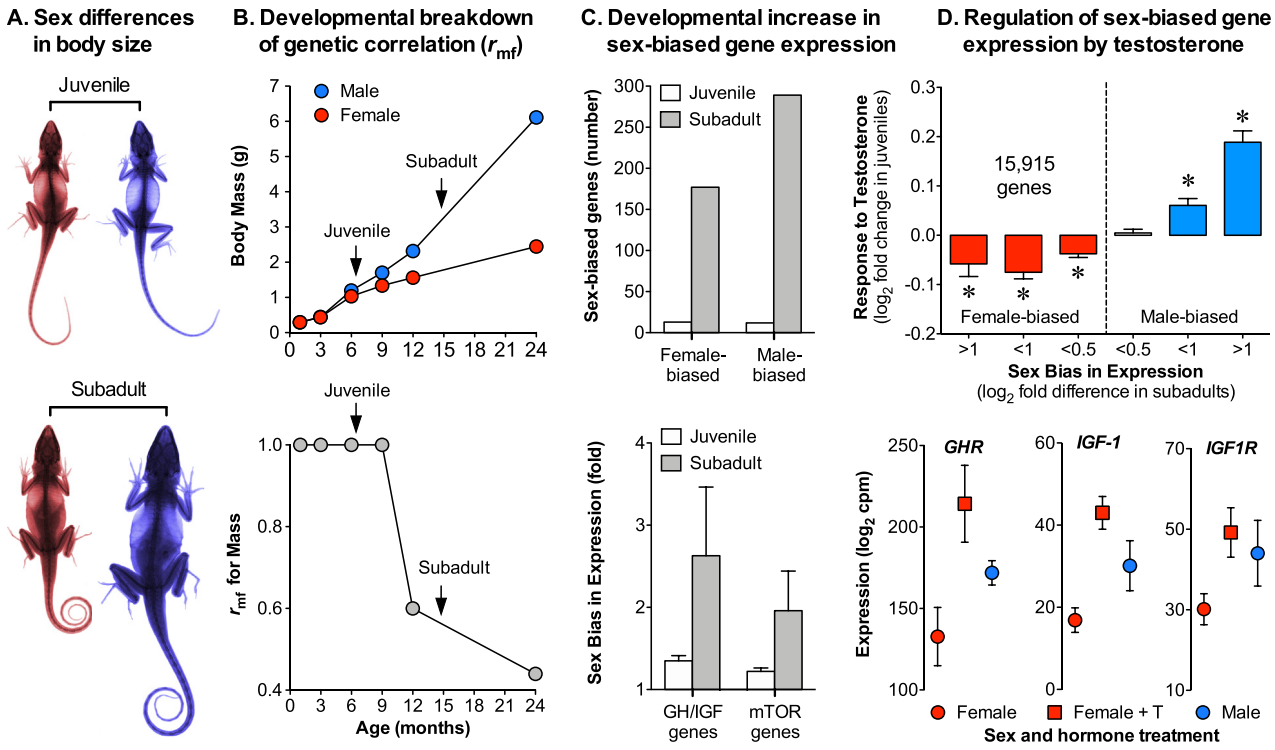

Fig. 3. (**A**) Radiographs of female (red) and male (blue) brown anoles illustrate the rapid development of sexual size dimorphism (SSD) from juveniles (top) to subadults (bottom). (**B**) The development of SSD in body mass (top) is associated with the breakdown of the between-sex genetic correlation (r_{mf}) for body mass (bottom). (**C**) The liver transcriptome shows a sharp developmental increase in the total number of significantly sex-biased genes (top) and in the mean (± SE) magnitude of sex-biased expression for genes in key growth-regulatory pathways such as GH/IGF and mTOR (bottom). (**D**) Across the entire liver transcriptome, genes that eventually become female-biased in subadults tend to be inhibited by treatment of juvenile females with testosterone (T), whereas those that eventually become male-biased in subadults tend to be stimulated by T (top). Treatment of juvenile females with T masculinizes expression of key growth-regulatory genes such as growth hormone receptor (*GHR*), insulin-like growth factor-1 (*IGF-1*), and its receptor (*IGF1R*) (bottom). Adapted from Cox et al. (2017b) in The American Naturalist.

pathways such as the growth hormone/insulin-like growth factor (GH/IGF) and mechanistic target of rapamycin (mTOR) pathways (Fig. 3C). Treatment of juvenile females with T induces male-like patterns of expression across the entire liver transcriptome by tending to upregulate those genes that eventually become male-biased during maturation and tending to downregulate those genes that eventually become female-biased (Fig. 3D). Similar effects are observed for key genes in the GH/IGF pathway (Fig. 3D), such as growth hormone receptor (*GHR*), insulin-like growth factors (*IGF-1, IGF-2*), IGF-1 receptors (*IGF1R*), and IGF binding proteins (*IGFBP1, IGFBP4*) (Cox et al., 2017b). The picture that emerges is one in which maturational increases in circulating T levels in males lead to sex differences in the liver transcriptome by altering the expression of key growth-regulatory genes that stimulate rapid growth in males, thereby causing the breakdown of r_{mf} for body size. A direct test of this hypothesis would require estimation of the between-sex genetic correlation for size in a situation where T is blocked or removed via castration, or where T is supplemented in females, which is currently in progress. From an evolutionary perspective, sex differences in selection on the size of juveniles would be unlikely to result in sex-specific evolution due to high r_{mf}, whereas sex differences in selection on adult size would be more likely to result in sex-specific evolution due to the reduced between-sex genetic correlation, which is orchestrated by sexual divergence in plasma T levels (Cox et al., 2017b). In this way, sex steroids can influence the genetic potential for phenotypic evolution.

5. Sex steroids as mediators of evolutionary transitions

Theoretical models for the evolution of sexual dimorphism via the breakdown of r_{mf} often view sex-specific adaptation as a relatively slow process in which the shared genetic basis for a trait is gradually diminished over long evolutionary timescales (Fairbairn and Roff, 2006; Lande, 1980, 1987). An alternative view is that major evolutionary transitions may be expedited simply by altering the ways in which genetic and physiological regulatory axes are functionally coupled to sex steroids, requiring minimal rearrangement of the networks and genes themselves.

The evolution of T-mediated seasonal singing in songbirds provides a potential example of how a conserved network of genes can be evolutionarily coupled to a hormonal signal to mediate an evolutionary transition (Fig. 4A–C). The structure of the brain's song control system is similar across species of songbirds, including those with little seasonal variance in singing behavior and song circuit morphology, such as the zebra finch (*Taeniopygia guttata*), and those that exhibit plasticity in song and circuit morphology across seasons, such as the canary (*Serinus canaria*). Whereas song structure is relatively insensitive to T in the zebra finch, many seasonally variable aspects of song structure are sensitive to T in the canary (Frankl-Vilches and Gahr, 2018). In HVC, a region of the song control system, treatment of male canaries with T induces many of the same patterns of gene expression that differentiate long-day (breeding season) from short-day (non-breeding) canaries (Frankl-Vilches et al., 2015). As with song itself (Fusani et al., 2003), these transcriptional effects of T are mediated through a combination of AR and ERα signaling following local conversion of T to E_2 by

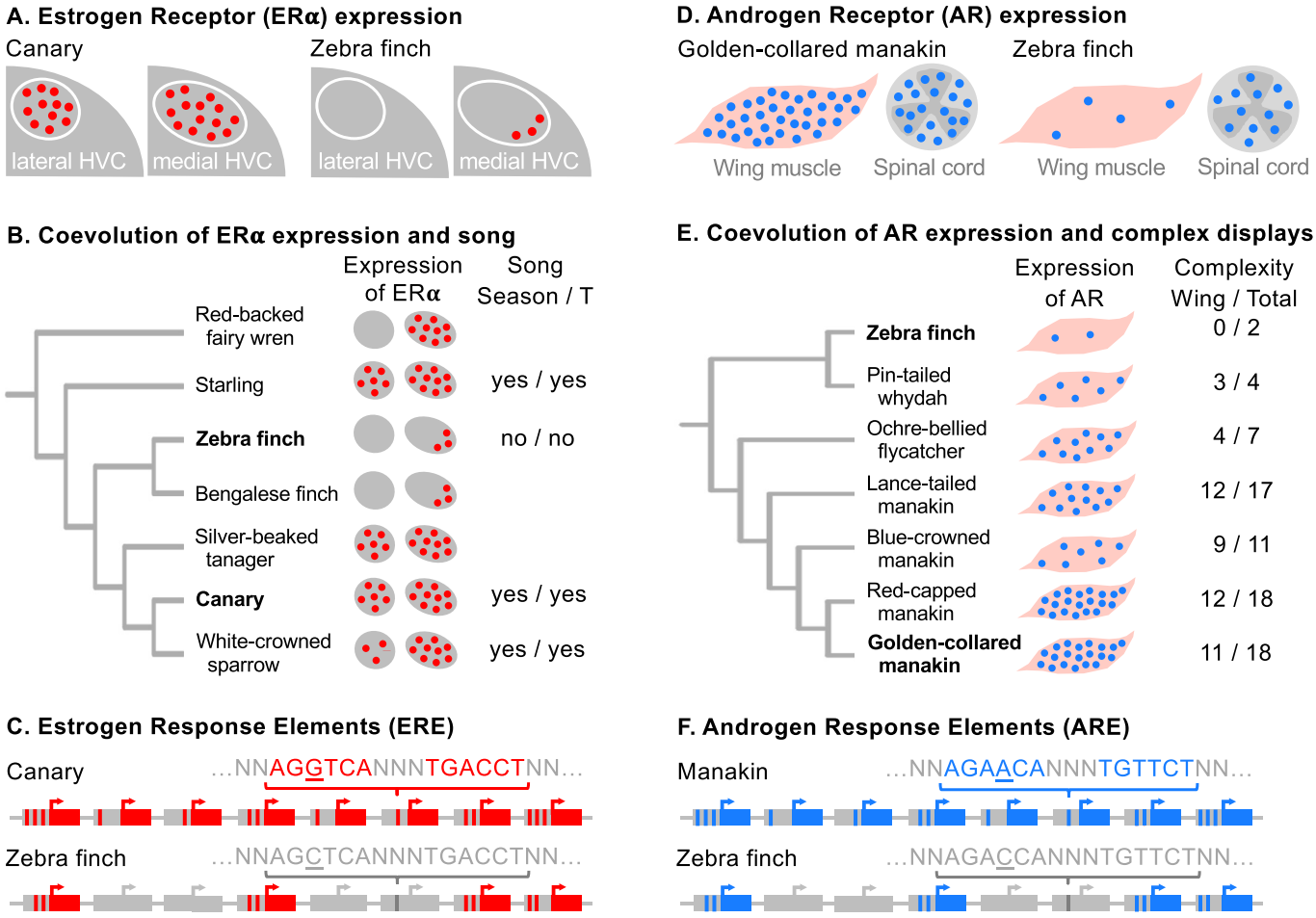

Fig. 4. Examples of evolutionary transitions in sexual signaling potentially mediated by shifts in ER expression and ERE distribution. (**A**) Simplified representation of differences in ER expression (red dots) in two brain regions of focal species with different song phenotypes. (**B**) Phylogenetic patterns in ER expression across 7 songbird species, with information whether song phenotypes are seasonal and T-mediated for several species. (**C**) Simplified representation of the gain/loss of ERE motifs due to point mutations (underscored) in promoter regions of genes that consequently gain (red) or lose (gray) estrogen-mediated expression, as inferred from genome comparisons of two focal species with different song phenotypes. (**A-C**) (**D**) Simplified representation of differences in AR expression (blue dots) in two tissues of focal species with different display phenotypes. (**E**) Increases in AR expression in wing muscle across 7 songbird species are correlated with increases in display complexity. (**F**) Simplified representation of the gain/loss of ARE motifs due to point mutations (underscored) in promoter regions of genes that consequently gain (blue) or lose (gray) androgen-mediated expression, as inferred from genome comparisons of two focal species with different display phenotypes. (**A-C**) Adapted from Frankl-Vilches and Gahr (2018) in Journal of Comparative Physiology A under a Creative Commons Attribution 4.0 International License (http://creativecommons.org/licenses/by/4.0/). (**D-F**) Illustrations based on results in Fuxjager et al. (2015) in Functional Ecology and Fuxjager and Schuppe (2018) in Journal of Steroid Biochemistry and Molecular Biology.

aromatase (Frankl-Vilches et al., 2015). Searches of the canary genome *in silico* reveal that genes with seasonal and T-responsive expression in HVC are enriched for putative ARE and ERE motifs within their promoter regions (Frankl-Vilches et al., 2015). However, 4–8% of these putative AREs and 20% of these putative EREs are absent from the zebra finch genome, due to a combination of nucleotide substitutions altering the motif (i.e., evolutionary gains or losses of an ARE or ERE, Fig. 4C) or complete absence of an orthologous promoter region (Frankl-Vilches et al., 2015). Though evolutionary inferences are limited in this two-point comparison across distantly (40 my) related species, these findings suggest that subtle point mutations can effectively alter the genomic locations of many hormone response elements, thereby coupling (or decoupling) genes from regulation by sex steroids and facilitating the evolution of seasonal song (Fig. 4C).

The observation that genes with seasonal and T-sensitive expression in the canary are more likely to have unique EREs than AREs is also consistent with broader comparative patterns of AR and ER expression. Across a variety of songbird species, AR mRNA and protein is always highly expressed in HVC, indicating that responsiveness to androgens is phylogenetically conserved for this brain region (Frankl-Vilches and Gahr, 2018). By contrast, patterns of ERα expression in HVC are highly variable across species (Fig. 4B), with canaries and other seasonally singing fringillids characterized by high ERα expression throughout HVC, whereas the zebra finch and other estrildids express only low levels of ERα in the medial HVC and lack ERα expression entirely in the lateral HVC (Frankl-Vilches and Gahr, 2018). Therefore, evolutionary shifts in both tissue-specific ERα expression throughout the organism and gene-specific ERE distribution throughout the genome likely facilitated the evolution of seasonal song (Fig. 4A–C).

The evolution of acrobatic courtship displays in manakins provides an analogous example, albeit with tissue-specific changes in AR expression and genomic ARE distribution playing a greater role than estrogenic signaling (Fig. 4D–F). In the golden-collared manakin (*Manakus vitellinus*), males court females by forcing their raised wings together to produce audible snaps and trills that can require wing movements at twice the maximal frequency used for powered flight (Fuxjager and Schuppe, 2018). Selective androgen inhibitors slow the rate of wing snaps (Fuxjager et al., 2013), and AR expression in several key wing muscles is required to achieve near-superfast levels of contraction and relaxation (Fuxjager et al., 2017). Across four manakin

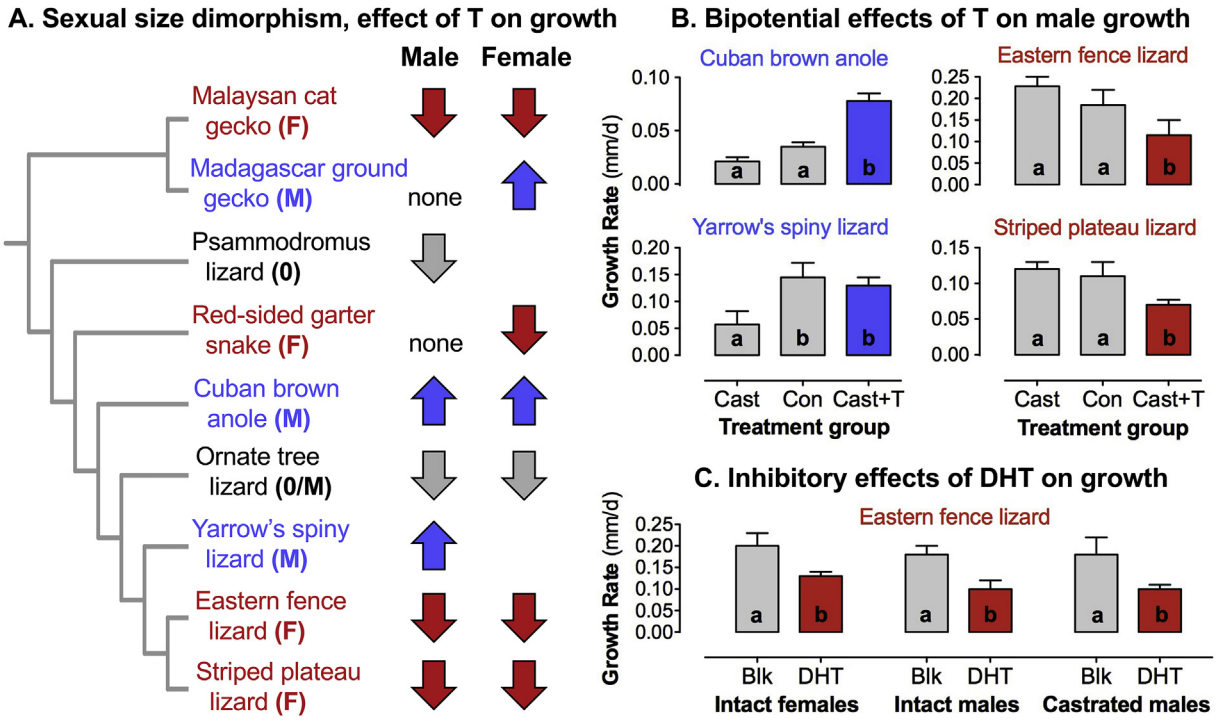

Fig. 5. (A) Evolutionary shifts in sexual size dimorphism (F = female-biased, M = male-biased, 0 = monomorphic, 0/M = weakly male-biased) are associated with shifts in effects of exogenous T on growth of males (left column) and females (right column). Arrows indicate inhibition (pointing up) or stimulation (pointing down) by T, none = no significant effect of T. Effects consistent with patterns of SSD are shown in red and blue, effects that are inconsistent are shown in gray. Literature sources are summarized in Table S1. (**B**) Bipotential effects of T on growth, as inferred from comparison of castrated males (Cast), intact control males (Con), and castrated males with T implant (Cast + T) across four species. Lowercase letters denote post hoc statistical separation. Colors indicate the groups receiving exogenous hormone. Data from Yarrow's spiny lizard and the Striped plateau lizard are adapted from Cox and John-Alder (2005) in Journal of Experimental Biology. Data from the Eastern fence lizard are adapted from Cox et al. (2005) in Physiological and Biochemical Zoology. Data from the Cuban brown anole are adapted from Cox et al. (2009a,b) in Journal of Evolutionary Biology. (**C**) Growth inhibition by 5α -dihydrotestosterone (DHT) relative to animals with empty implants (Blk) implicates AR signaling in growth inhibition by T. Adapted from Pollock et al. (2017) with permission from N. Pollock, H.B. John-Alder, and the Journal of Experimental Biology.

species with relatively complex displays and three other songbird species with less complex displays (Fig. 4E), the evolution of increased motor complexity in wing movements and displays is positively correlated with the degree of AR (but not ER) expression in key wing muscles (Feng et al., 2010; Fuxjager et al., 2015). Treatment of golden-collared manakins with T induces the expression of genes that influence muscle fiber contractility, shorten muscle relaxation times, and support muscular hypertrophy (Fuxjager et al., 2012, 2016; Fuxjager and Schuppe, 2018). T also promotes a more robust transcriptional response in wing muscle of the golden-collared manakin than it does in that of the zebra finch, which does not use wing displays in courtship (Fuxjager et al., 2016). Although this is likely due in part to the lower levels of AR expression in zebra finch muscle (Fig. 4D), *in silico* comparison of the genomes for each species reveal substantially more ARE motifs in the promoter regions of manakin genes, relative to their zebra finch orthologs (Fuxjager et al., 2016). Therefore, evolutionary shifts in both tissue-specific AR expression and gene-specific ARE distribution may have facilitated the evolution of complex wing movements and mating displays in manakins (Fig. 4D–F).

Genetic differences in AR and ER expression can also segregate within populations, as in the case of white-throated sparrows (*Zonotrichia albicollis*), which occur in two genetic morphs. White-striped morphs are typically heterozygous for a large, non-recombining chromosomal inversion ($ZAL2^m$) and engage in more territorial aggression and mate-finding behavior than tan-striped males, which are homozygous for the ancestral ZAL2 haplotype and prioritize mate guarding and nestling provisioning (Horton et al., 2014; Maney et al., 2015). Although white-striped morphs have higher circulating T and E_2 levels than do tan-striped individuals, morph differences in behavior persist even when T levels are experimentally equalized (Maney et al., 2015). This morph difference in hormonal sensitivity is likely due to corresponding differences in ERα expression that arise from alternative alleles of the *ESR1* gene, which encodes ERα and is captured within the $ZAL2^m$ inversion. A variety of fixed nucleotide substitutions, insertions, and deletions differentiate the ZAL2 and $ZAL2^m$ alleles of the *ESR1* promoter, resulting in predicted *in silico* differences in binding of key transcription factors, *in vitro* differences in transcription of ERα in cell culture, and *in vivo* morph differences in the expression of ERα in relevant brain regions of free-living birds (Horton et al., 2014). Moreover, levels of ERα expression in these brain regions predict individual variation in territorial aggression and parenting better than color morph alone (Horton et al., 2014; Maney et al., 2015). Collectively, these findings illustrate that genetic divergence in the regulation and expression of nuclear hormone receptors can occur over relatively short evolutionary timescales (i.e., within populations), facilitating divergence in associated behavioral phenotypes via hormonal pleiotropy.

The examples above illustrate how evolutionary transitions in song, courtship, and territorial behavior may be facilitated by genetically based shifts in tissue-specific AR and ER expression, and/or by genetic gain or loss of ARE and ERE motifs in regulatory regions of target genes that recognize these receptors as transcription factors (Fig. 4). In essence, these genetic changes result in the evolutionary coupling (or decoupling) of genes and phenotypes to (or from) regulation by androgens and estrogens. Other evolutionary transitions reflect similar types of adaptive hormonal coupling. Across six species of *Anolis* lizards, increased use of the forelimbs for stereotyped push-up displays is associated with increased AR expression in biceps muscle (Johnson et al., 2018b). Likewise, the evolution of androgen-mediated "foot-flagging" displays in the Bornean rock frog (*Staurois parvus*) is accompanied by increased AR expression in leg muscle, relative to other frogs

that rely solely on vocalization to attract mates (Mangiamele et al., 2016). Comparative evidence for functionally relevant gains and losses of ARE and ERE motifs is less common, in part because it requires comparison of detailed genomic information across multiple species (Frankl-Vilches and Gahr, 2018). However, targeted studies of individual species can provide clues about major evolutionary transitions. For example, developmental genetic studies in the little skate (*Leucoraja erinacea*) suggest that the evolution of internal fertilization in early vertebrates occurred via the acquisition of ARE motifs that modified the *Sonic hedgehog* (*Shh*) pathway to produce paired copulatory appendages (i.e., claspers) from pelvic fins in androgen-dependent fashion (O'Shaughnessy et al., 2015).

6. Bipotential sex steroids as mediators of evolutionary reversals

In the examples described above, the effects of sex steroids are consistently stimulatory (e.g., T stimulates song, courtship displays, territorial aggression, mate searching, push-ups, and "foot-flagging" displays). Consequently, evolutionary transitions are typically achieved either by strengthening (coupling) or weakening (decoupling) this unidirectional link between hormone and phenotype. A related possibility is that evolutionary transitions can be achieved by reversing the directional effects of hormones on phenotypes, as inferred from the observation that T is "bipotential" as both a promoter and an inhibitor of growth in reptiles (Fig. 5). Androgens such as T are classically regarded as promoters of skeletal elongation and overall organismal growth. This generalization is derived from a variety of model species in agriculture (e.g., pigs, cattle, turkey), aquaculture (e.g., tilapia, salmon), and medicine (e.g., rodents, primates), but primarily those in which males are the larger sex (Cox and John-Alder, 2005; John-Alder and Cox, 2007; John-Alder et al., 2007). In squamate reptiles (lizards and snakes), evolutionary reversals from male-to female-biased sexual size dimorphism (SSD) are common (Cox et al., 2007), but circulating levels of T are consistently higher in maturing and breeding males than in females, regardless of species or pattern of SSD (Cox and John-Alder, 2005; Cox et al., 2005; John-Alder et al., 2007). Although T has a classic stimulatory effect on growth in several squamate species in which males are the larger sex (Cox et al., 2009a, 2015; Cox and John-Alder, 2005), it also has a novel, inhibitory effect on growth in several species in which females are the larger sex (Abell, 1998; Cox and John-Alder, 2005; Cox et al., 2005; Lerner and Mason, 2001). This pattern suggests that evolutionary reversals in SSD may have been achieved, at least in part, by evolutionary reversals in the effects of T on growth (Fig. 5A).

In basal squamates such as geckos, E_2 and other ovarian hormones have been proposed as the primary endocrine mechanisms underlying sex differences in growth and SSD (Kubička et al., 2013, 2017; Starostová et al., 2013). In part, this idea follows from concerns that the high levels of circulating T that are sometimes induced by exogenous implants may exert their effects by suppressing normal ovarian function and/or after conversion to E_2, rather than via physiologically relevant AR signaling (Kubička et al., 2013, 2017; Starostová et al., 2013). Indeed, instances of growth inhibition by T have been observed in situations that are not predicted by patterns of SSD (Fig. 5A), and coincide with concerns about pharmacological T levels (Hews et al., 1994; Hews and Moore, 1995; Lerner and Mason, 2001). Nonetheless, exogenous T inhibits growth of males and females in a gecko with female-biased SSD (Kubička et al., 2013), and ovariectomized females treated with T or DHT grow more quickly than intact females and attain sizes typical of males in a gecko species with male-larger SSD, though it is not possible to disentangle the effects of androgens from those of ovariectomy in this example (Kubička et al., 2017). DHT inhibits growth in both sexes of the eastern fence lizard (*Sceloporus undulatus,* Fig. 5C), a species with female-biased SSD (Pollock et al., 2017), whereas DHT stimulates growth in the Cuban brown anole (*Anolis sagrei*), a species with male-biased SSD (A. Walsh, T. Wittman, and R.M. Cox, unpublished data). These findings implicate AR signaling in both inhibition and stimulation of growth by T. Limited evidence supporting bipotential growth regulation is also available from other lineages. Whereas T and DHT stimulate growth in turkeys (Fennel and Scanes, 1992a), these same androgens inhibit growth in chickens (Fennel and Scanes, 1992b). Likewise, exogenous T administered in the yolk of developing eggs stimulates postnatal growth in many birds (Navara et al., 2005, 2006; Pilz et al., 2004; Schwabl, 1996), but this same embryonic treatment tends to inhibit postnatal growth in raptors with "reversed" SSD, including the American kestrel (*Falco sparverius*) and the Eurasian kestrel (*F. tinnunculus*) (Fargallo et al., 2007; Sockman and Schwabl, 2000; Sockman et al., 2008).

Although the evidence for bipotential growth regulation by T is somewhat limited due to sparse phylogenetic sampling (Fig. 5A) and differences in methodology and interpretation across studies (Starostová et al., 2013), it nonetheless raises intriguing questions of how such reversals may be achieved at physiological and genetic levels. Until recently, the lack of a toolkit for molecular endocrinology and comparative genomics in reptiles has made it difficult to address these questions. However, in the brown anole (*Anolis sagrei*), it was recently shown that the increase in male growth that gives rise to male-biased SSD occurs coincident with a sharp decrease in hepatic *SHBG* transcript (potentially increasing the availability of free androgens) and a dramatic increase in the expression of insulin-like growth factors (*IGF-1, IGF-2*) in males (Cox et al., 2017b). Similar expression patterns in these growth-regulatory genes can be induced by T (Fig. 3D), which is consistent with growth promotion by T in this species (Fig. 5B). By contrast, the growth-inhibitory effects of T in the eastern fence lizard (*Sceloporus undulatus*, Fig. 5B) are associated with T-mediated decreases in *IGF-1* expression, as assessed by RT-qPCR (Duncan, 2011). Preliminary liver transcriptomes from *S. undulatus* reveal that, as in *A. sagrei*, exogenous T decreases the expression of *SHBG,* potentially increasing the availability of free T. However, in contrast to *A. sagrei,* T decreases the expression of *IGF-1* and *GHR* in this species with female-biased SSD (C.L. Cox, D. Card, T.A. Castoe, N. Pollock, H.B. John-Alder, R.M. Cox, in prep). Ongoing work is comparing sex-biased and T-mediated gene expression across closely related *Sceloporus* species with divergent patterns of SSD, and results to date suggest that patterns of both sex-biased and T-mediated gene expression differ substantially between species. Expansion of this comparative approach across replicate evolutionary changes in SSD (Fig. 5A), coupled with the recent availability of a high-quality genome assembly for the eastern fence lizard, should greatly improve our understanding of the evolution of bipotential growth regulation by androgens. In particular, it will be interesting to test whether evolutionary gains, losses, or shifts in the location of ARE motifs within the regulatory regions of T-mediated genes in the eastern fence lizard (e.g., relative to the *Anolis* genome as a representative male-biased lizard) can explain the repeated evolution of "atypical" growth inhibition by androgens in this and other squamate lineages.

Declaration of competing interest

The author has no competing interests to declare.

Acknowledgements

Many of the ideas in this paper grew out of collaborations and discussions with F. Bonier, R. Calsbeek, C.L.Cox, H.B. John-Alder, and J.W. McGlothlin. Thanks to C.D. Robinson, A. Walsh, and T. Wittman for providing unpublished data. All vertebrate research conducted by the author that is summarized in this review was done in accordance with scientific collecting permits from relevant states and countries (as reported in the original publications) and with approval from institutional Animal Care and Use Committees, particularly the University of Virginia ACUC (protocol 3896). Much of the research that is

summarized in this review was conducted with financial support from the US National Science Foundation (DEB-1453089, IOS-1755026, and IOS-1539936 to RMC) and from the University of Virginia.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.mce.2019.110668.

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