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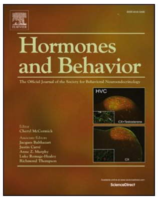

Activational vs. organizational effects of sex steroids and their role in the evolution of reproductive behavior: Looking to foot-flagging frogs and beyond☆

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ABSTRACT

Sex steroids play an important role in regulation of the vertebrate reproductive phenotype. This is because sex steroids not only activate sexual behaviors that mediate copulation, courtship, and aggression, but they also help guide the development of neural and muscular systems that underlie these traits. Many biologists have therefore described the effects of sex steroid action on reproductive behavior as both "activational" and "organizational," respectively. Here, we focus on these phenomena from an evolutionary standpoint, highlighting that we know relatively little about the way that organizational effects evolve in the natural world to support the adaptation and diversification of reproductive behavior. We first review the evidence that such effects do in fact evolve to mediate the evolution of sexual behavior. We then introduce an emerging animal model – the foot-flagging frog, *Staurois parvus* – that will be useful to study how sex hormones shape neuromotor development necessary for sexual displays. The foot flag is nothing more than a waving display that males use to compete for access to female mates, and thus the neural circuits that control its production are likely laid down when limb control systems arise during the developmental transition from tadpole to frog. We provide data that highlights how sex steroids might organize foot-flagging behavior through its putative underlying mechanisms. Overall, we anticipate that future studies of foot-flagging frogs will open a powerful window from which to see how sex steroids influence the neuromotor systems to help germinate circuits that drive signaling behavior. In this way, our aim is to bring attention to the important frontier of endocrinological regulation of evolutionary developmental biology (*endo*-evo-devo) and its relationship to behavior.

1. Introduction

Reproductive behavior in animals is as diverse as it is spectacular. This is especially true with respect to behavioral traits that help animals compete with rivals and court potential mates. But, how do new behavioral traits arise in the first place, and how do they evolve to such extreme ends? Answers to these questions are multifaceted, and they have been the focus of study of several fields of biology for decades (Ryan, 2021; Westneat et al., 2010). Most often, those who study the evolution of reproductive behavior do so in ecological and/or functional contexts. Iconic examples include studies that explore why male bowerbirds construct large bowers to attract mates (Borgia, 1995; Kusmierski et al., 1997; Uy and Borgia, 2000), or how complex head-bobbing displays evolve in *Anolis* lizards (Ord et al., 2001, 2002). Other fascinating work in this area centers on the evolution of alternative reproductive tactics (Bailey et al., 2010; Wolff and Cicirello, 1990) and the factors that mediate divergence in mating systems (Anholt et al., 2020; Bowyer et al., 2020). However, we should also move outside of the

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ecological/functional context when considering the evolution of such traits, and instead focus on them from a mechanistic or physiological point of view (Bass and Chagnaud, 2012; Fischer and O'Connell, 2017; Fuxjager and Schlinger, 2015; Hoke et al., 2019; Jourjine and Hoekstra, 2021; Schwark et al., 2022). This approach is equally essential to understanding the evolution of reproductive behavior because the neural and neuromuscular systems that underlie reproduction set the stage on which selection acts to influence how the reproductive phenotype (including its behavioral underpinnings) change through time.

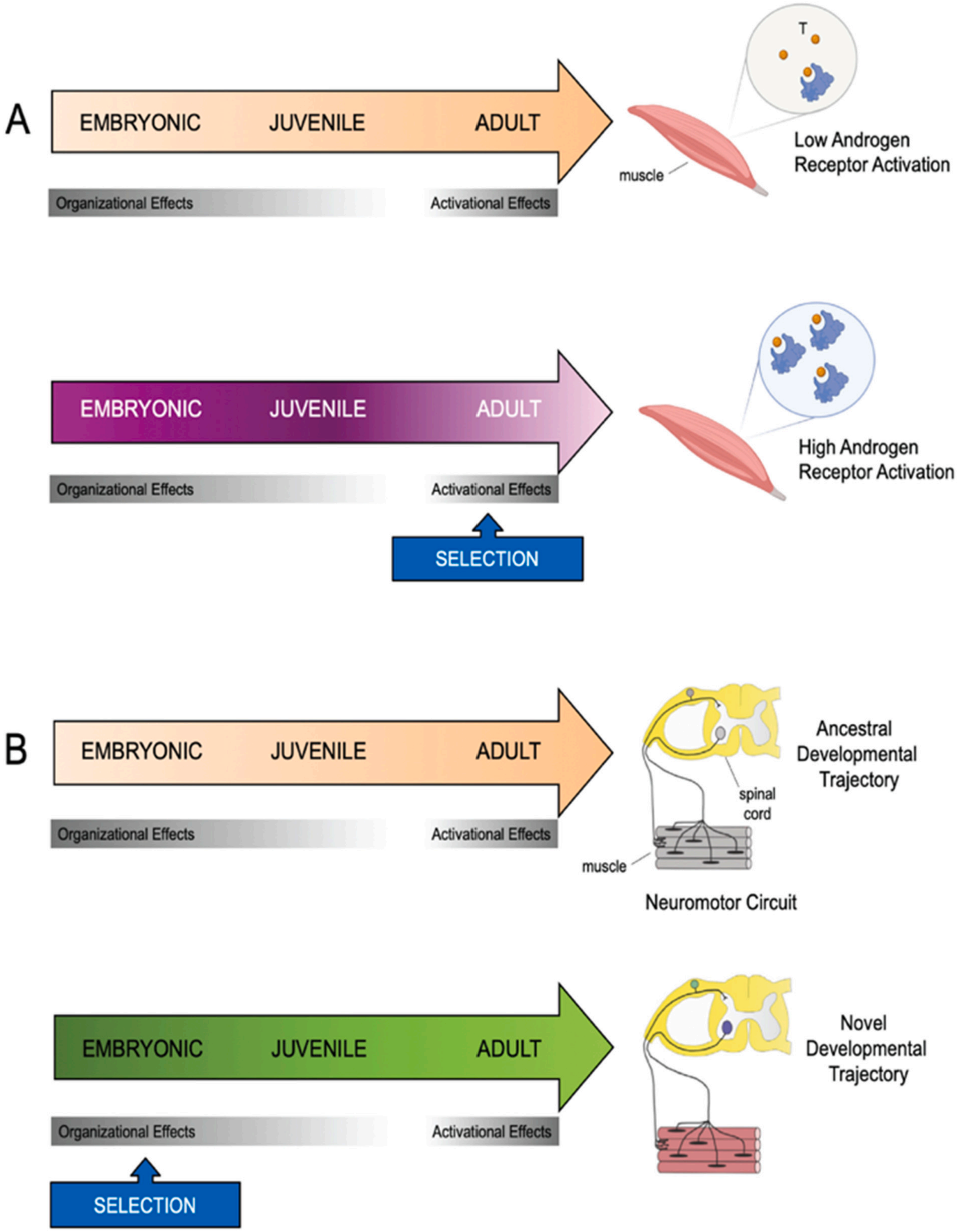

Fig. 1. Selection may act on the activational effects of hormones, for instance to increase androgen receptor activation in muscle tissue (A), which may "dial up" the effects of hormones to promote novel behaviors. Alternatively, or in addition, selection may act on the organizational effects of hormones to influence the developmental trajectory of an androgen-sensitive neuromotor pathway (B), which could enable a novel behavior to emerge.

Consider sex steroids, which are integral to the evolution of vertebrate reproductive behavior (as well as other traits outside the realm of reproduction). This class of hormone, which includes androgens, estrogens, and progestins, mediates much of the behavior associated with copulation, courtship, and sexual aggression (Adkins-Regan, 2005; Crews and Moore, 1986). Thus, if selection alters any of these traits, then it must also modify underlying sex steroid systems. One factor complicating this process is that steroid hormones themselves are highly conserved with respect to their molecular structure (Schuppe et al., 2020). In other words, a steroid hormone like the androgen testosterone (T) is identical in most species (however, in the case of T, fish are a notable exception because they have a slightly different bioactive androgen called 11-ketotestosterone, or 11-KT) (Adkins-Regan, 2005). This means that selection does not likely alter reproductive behavior by influencing steroid hormone structure, but instead influences behavior by altering other facets of the machinery that underlie steroid hormone signaling (Hau, 2007).

In recent years, biologists have begun to take a deep dive into the mechanisms by which sex steroid systems evolve to facilitate behavioral adaptation and diversification (Adkins-Regan, 2008; Cox, 2020; Fuxjager et al., 2018; Fuxjager and Schuppe, 2018; Hau, 2007; Ketterson et al., 2009; Lipshutz et al., 2019). This work largely focuses on how selection might change the way sex steroids are detected in a target tissue, and how the effects of sex steroid action are transduced. Researchers have predominantly used adult animals for this work, and thus have considered the so-called "**activational**" effects of steroid hormone action, which refer to the ability of steroids to "dial up" or "dial down" behavioral output. Although this approach can reveal core insights about how steroid systems evolve, it does not shed light on all the other ways that sex steroids can regulate behavior. Behavioral traits are also shaped by processes that occur early in life and that have long-lasting consequences. Here, we mean the "**organizational**" effects of sex steroids on reproductive traits, which in the case of behavior refers to the role that steroid action plays during development to help shape neural circuits that are then activated during adulthood to produce reproductive behavior. The relationship between these two processes has long driven research in the field of behavioral endocrinology, and continues to this day to do so (Adkins-Regan, 1983; Adkins-Regan, 2012; Arnold, 2009b; Arnold and Breedlove, 1985; Beatty, 1979; Goy and Phoenix, 1972; Phoenix et al., 1959). If modifications to sex steroid signaling machinery underlie the evolution of reproductive behavior, then we might expect these effects to occur at two levels—during and after development (See Fig. 1).

In the current paper, we explore the idea raised above more thoroughly. We begin by providing a basic primer of activational vs. organizational effects of sex steroids on animal sexual behavior. We explore the literature that suggests that sex steroid signaling machinery underlies behavioral adaptation, while highlighting why we know that many of these effects arise through developmental processes. We end with a deeper exploration of an emerging animal model—the Bornean rock frog (*Staurois parvus*)—that might shed some light on this issue. This species has evolved a novel gestural waving signal called the "foot flag," which it uses to compete with rivals for access to mates (Hödl and Amézquita, 2001; Preininger et al., 2013b). When males are given exogenous T, they begin to foot flag more frequently and produce displays with a rounder shape (Anderson et al., 2021a; Mangiamele et al., 2016). Moreover, studies show that the foot flag's evolution is marked by a significant increase in androgen receptor (AR) expression in the thigh muscles that actuate waving movements (Anderson et al., 2021c; Eigerman and Mangiamele, 2022; Mangiamele et al., 2016; Smith et al., 2021). Frogs are widely used to study developmental processes, largely because they undergo a metamorphic change from tadpole to frog. In doing so, researchers can probe the mechanisms that make this transition happen. Here, we compare select behavioral, endocrine, and neural traits between juvenile and adult *S. parvus* in an attempt to disentangle the effects of muscular AR on display production during adulthood from behavioral mechanisms that are likely innate or that arose during a critical developmental moment in the frog's life. Overall, our aim is to begin to understand how selection can drive the evolution of the reproductive phenotype by altering not only how hormones mediate processes in the nervous system, but also how hormones help set up the nervous system in the first place.

2. Activational and organizational effects of sex steroids

Our understanding of sex steroids and their ability to activate reproductive behavior is based on extensive data sets that span a wide range of taxa (reviewed in Adkins-Regan, 2005; Nelson and Kreiegsfeld, 2015). In some ways, activational studies are more straightforward to conduct, compared to organizational ones, because they often involve manipulations within adult animals. As a result, we have constructed a clearer understanding of how androgens, estrogens, and progestins act within an organism to increase the probability that they express certain behavioral traits (though, it is important to note that this work is done primarily in males, which means that we do still lack in our understanding of how these hormones activate female reproductive behavior). In fact, many think about sex steroid action in this regard, referring to it as a mechanism to coordinate internal physiology with the external environment (Adkins-Regan, 2005; Adkins-Regan, 2012; Ball and Balthazart, 2008; Crews and Moore, 1986; Williams, 2008; Young and Crews, 1995). In less abstract terms, we might describe this phenomenon as the ability of sex steroid action to help mediate the context-appropriate expression of reproductive behavior. Such effects ensure, for example, that traits like courtship displays are produced at the right time of year, and in the correct way. The same principle applies for the other reproductive traits whose functions depend on correct spatial and temporal expression (aggression, copulation, etc.).

From an evolutionary perspective, we can gain important insight into the evolution of hormone-behavior relationships through comparative studies. Particularly important are studies that look at closely related taxa, which have strongly diverged with respect to prominent reproductive traits and their hormonal correlates (Fink et al., 2006; Garrick and Lang, 1977; Ginsberg and Rubenstein, 1990; Miles and Fuxjager, 2018; Ord et al., 2001). In *Peromyscus* mice, for instance, studies show that monogamous and territorial male California mice (*P. californicus*) release T after winning aggressive encounters, and this hormone then acts in the brain to promote future territorial aggression (Fuxjager et al., 2010; Fuxjager et al., 2011b; Oyegbile and Marler, 2005). However, things are quite different in the closely related white-footed mouse (*P. leucopus*), which is promiscuous and non-territorial. In this latter species, males do not release T after winning a fight, and the experience of victory does not act to increase the odds of winning future contests (Oyegbile and Marler, 2006). Thus, there is a clear species difference in terms of how T is released after a fight, as well as T's effect on territorial aggression. However, if one phenotypically engineers a post-encounter T pulse in white-footed mice so that it resembles that of a California mouse, then white-footed mice also develop a winner effect (i.e., they are more likely to win fights in the future) (Fuxjager et al., 2011a). This result suggests that the neural systems necessary for T-dependent territorial aggression are likely present in white-footed mice, even if these systems are not routinely activated by T release immediately following a male-male encounter. Divergence in the two species' behavior therefore likely occurs in part through the dissociation of T and aggression in white-footed mice, or through the linking of T and aggression in California mice (or both). In either case, this would be an example of evolution in the activational effects of hormones on behavior (although, we recognize that organizational effects may still also play a role).

Our conceptualization of steroid hormones and their ability to "organize" the brain is largely framed by our knowledge of sexual differentiation. We can define sexual differentiation as the pathways by which sex differences in anatomy, physiology, neurobiology, and behavior arise during development (Adkins-Regan, 2005, 2008; Adkins-

Regan, 2012; Arnold, 2009b; Arnold and Breedlove, 1985; McCarthy, 2016). Unsurprisingly, sexual differentiation is highly complex, varying among mammals, birds, reptiles, amphibians and fish (Manolakou et al., 2006). There are several reviews that explore these differences and recognize the myriad factors that play a role in shaping how sex differences emerge. These pathways, however, are beyond the scope of this paper per se; rather, our aim is to point out that sex steroids are vital to this process in all these taxa, even if the precise mechanisms of their contribution differ (Nakamura, 2010). In mammals, for example, we know that the *SRY* gene on the Y chromosome functions as the genetic trigger that sets off the development of the gonads, which in turn create and release T (Koopman et al., 1991; Sekido and Lovell-Badge, 2009). When T circulates in a developing embryo, it helps create a neural phenotype that leads to the expression of "male-typical" behavior during adulthood (Auger, 2004; McCarthy, 2016). By contrast, in most reptiles, sex is determined largely by temperature, as opposed to specific genes on a sex chromosome (Sarre et al., 2004). For such animals, it appears that temperature (either directly or indirectly) mediates the ability of sex steroids to drive gonadal differentiation. Birds, amphibians, and fish similarly vary in terms of sexual differentiation and its mechanistic basis, yet again sex steroid action is integral to the process (Elbrecht and Smith, 1992; Hayes, 1998; Kobayashi and Nagahama, 2009).

Additional work that reveals the complexities of sexual differentiation comes from studies that use the "four core genotypes" mouse model (Arnold and Chen, 2009; De Vries et al., 2002). In these mice, the SRY gene is removed from the Y chromosome and placed on an autosomal chromosome. This manipulation provides XX and XY gonadal males (i.e., individuals with a male-typical hormone profile during development), as well as XX and XY gonadal females (i.e., individuals with a female-typical hormone profile during development). Researchers can then use these animals to compare the effects of an XX or XY genotype in individuals with the exact same gonadal phenotype. As a result, organizational effects that are due to sex steroid hormone action early in life can be disentangled from the effects that are due to other genetic factors (i.e., expression of genes on the sex chromosomes). It turns out that many traits are in fact organized by sex steroid action early in development (Arnold, 2009a; Arnold and Chen, 2009); however, there is also a wide range of traits are influenced by genetic factors related to genomic sex (De Vries et al., 2002; Gatewood et al., 2006; Gioiosa et al., 2008; McPhie-Lalmansingh et al., 2008). In some cases, we even see complex interactions between sex steroids and genomic sex on the resulting phenotype.

3. Evolution of steroid signaling systems for behavioral diversification

How does selection influence the evolution of a steroid signaling system? Many have considered this question, particularly on theoretical grounds (Adkins-Regan, 2008; Cox, 2020; Fuxjager et al., 2018; Fuxjager and Schuppe, 2018; Hau, 2007; Ketterson et al., 2009; Lipshutz et al., 2019). Indeed, this topic taps into broader discussions about molecular system evolution and its effects on the phenotype (e.g., Emlen et al., 2012; Jin and Pawson, 2012; Pires-daSilva and Sommer, 2003). Recall from the introduction that most steroid molecules are highly conserved; for example, T in birds is identical to T in mammals. There are a few exceptions to this rule, but the general implication is that selection cannot influence mechanisms of steroid hormone action by altering steroid hormones themselves. Rather, selection is more likely to change how steroid systems work by modifying *i*) the molecular pathways that detect and transduce steroidal effects on a target cell, and *ii*) the biochemical pathways of steroid synthesis and/or breakdown (Fuxjager and Schuppe, 2018; Hau, 2007). There is still some debate about the degree to which different components of steroid signaling cascade can evolve independently of the others (see references above); however, there is growing recognition that the steroid signaling systems do appear to evolve in a tissue-specific manner at least in some cases to mediate behavioral adaptation and/or diversification. Either way, both routes of change are applicable to evolution of how sex steroids regulate the activation and organization of behavior.

To date, some of the strongest support for the framework described above comes from studies that attribute behavioral variation within and across species to tissue-specific expression patterns of sex steroid receptors. Field studies in a small bird called the dark-eyed junco (*Junco hyemalis*), for example, show that individual differences in territorial behavior are positively associated with such differences in the relative abundance with which AR and estrogen receptor (ER) are expressed in parts of the amygdala and hypothalamus (Rosvall et al., 2012). Other studies in a population of male spotted antbirds (*Hylophylax n. naevioides*) show that both amygdalar AR and hypothalamic ER (particularly in the preoptic area) increase in the non-breeding season to support territorial aggression when circulating levels of T are low (Canoine et al., 2007). Again, these studies together suggest that individuals that show higher levels of territorial aggression also maintain specific brain nuclei that are more sensitive to the effects of androgenic and estrogen action. Assuming such aggression is adaptive, then selection for territorial behavior may occur by driving the evolution of increased AR and ER in brain areas like the amygdala and preoptic area, or other areas that play a part in the regulation of sociosexual interactions (see also the 'evolutionary potential hypothesis'; reviewed in Hau, 2007).

Similar evidence comes from species comparisons. In lizards, for instance, species differences in the intensity of territorial push-up displays are positively associated with species variation in AR expression in the forelimb muscles of adult males (Johnson et al., 2018). Likewise, work in manakin birds shows that the complexity of their acrobatic courtship displays is positively linked to AR levels in males' wing muscles (Fuxjager et al., 2015). This latter study also shows that such a relationship does not exist in other levels of the motor system, nor does it exist in the context of other steroid hormone receptors such as ER. Indeed, there are several other examples that similarly attribute the evolution of displays to AR levels in muscle, even if they do not employ a broad phylogenetic approach to the analysis (reviewed by Tobiansky and Fuxjager, 2020).

The significance of the species comparison studies can be difficult to clarify, but experiments that directly test effects of AR in muscle can provide a guiding light to why selection might favor androgenic sensitivity in these tissues to support behavioral evolution. With respect to manakins, for example, studies show that inhibition of AR not only reduces the frequency with which males perform their courtship displays, but it also slows the speed at which males can produce these displays (Fusani et al., 2007; Fuxjager et al., 2013). In this way, we can imagine that selection for high performance displays proceeds through the concomitant evolution of increased androgenic sensitivity in specific target tissues (muscles) because it helps ready muscles for such behavior. Support for this idea comes from additional physiological work that demonstrates that AR mediates the expression of genes in these same muscles that encode proteins critical for calcium handling and flux within the myocyte (Fuxjager et al., 2012; Fuxjager et al., 2016a; Pease et al., 2022). The result is an AR-dependent increase in muscular contraction velocity and twitch times that "speed up" display behavior (Fuxjager et al., 2017; Tobiansky et al., 2020).

Thus far, we have largely considered how differences in behavior might arise through tissue-specific changes in AR and ER. But, it is equally important to remember that most vertebrates have several different types of receptors for androgens and estrogens (Guerriero, 2009). This means that there are likely a wide range of possible routes by which receptor-derived variation in behavior can evolve. Indeed, a tissue-specific change in the level at which any one sex steroid receptor is expressed may confer a concurrent shift to how an animal interacts with its physical and social environment. In addition, tissues can differ in terms of their ability to metabolize androgens and estrogens (Bentz et al., 2019; Fuxjager et al., 2016b; London et al., 2006; London and Schlinger, 2007; Newman et al., 2008; Pradhan et al., 2010; Rosvall et al., 2016; Schuppe et al., 2022). Such effects can relate both to a tissue's ability to make/process bioactive steroids or break down these hormones, which can augment and buffer steroidal effects on a target tissue, respectively (Demas et al., 2007; Soma et al., 2008). Perhaps the most extreme form of this phenomenon comes from work suggesting that certain tissues can make their own steroid hormones, because these comprising cells express all the transporters and enzymes necessary to convert cholesterol into either an androgenic or estrogenic hormone. We see evidence of such physiology in nuclei that control the oscine song system (London et al., 2006), as well as the skeletal muscles that actuate territorial drumming behavior in woodpeckers (Schuppe et al., 2022).

Importantly, many of the experiments outlined above occur in adult individuals and again focus on activational properties of steroid hormone action. But it is crucial to remember that androgens and estrogens may also be acting on neural systems that are laid down during development. Thus, selection on behavior may also occur on the organizational effects of sex steroids that shape these systems early in life, as the organism in question develops. Documenting these effects can be a challenge because it seemingly requires manipulations of individuals early in life. However, an alternate approach can be to manipulate the steroids that modulate a sexually-differentiated behavior in both male and female adult animals. Chiver and Schlinger (2017a, 2017b, 2019) elegantly use this tactic in golden-collared manakins (*Manacus vitellinus*), which is part of the avian family described above in which muscular AR likely evolves to support acrobatic display behavior. Researchers implanted female birds with T, which normally maintain low levels of this hormone, to test whether they begin to produce any male-typical courtship displays. The logic behind these experiments is that T in females *should* activate behaviors that do not require any sort of organizational effect, since the underlying systems for these behaviors would be in place in both sexes. If T fails to activate a given male-typical behavior in the female, then this particular behavior may be the result of an organizational effect possibly due to sex steroid action occurring early in life when sexual differentiation transpires. The results are compelling: T-treated female manakins show only part of the male-typical display (Chiver and Schlinger, 2017b). In other words, females given T produce certain gestural signals that males normally use for courtship, but females do not perform the full male courtship dance along the forest floor. Moreover, T-treated females begin to generate male-like vocalizations, but the acoustic structure of these calls is different than an adult male (Chiver and Schlinger, 2019). Finally, T treatment fails to cause females to build courtship areas, whereas the hormone does in males (Chiver and Schlinger, 2017a). Altogether, this work strongly suggests that certain parts of the golden-collared manakin display likely arise through organizational effects that occur before birds are reproductively mature. This work also shows, however, that some components of the display are controlled by mechanisms that occur in both males and females, and thus are likely generated through evolution that favors either the presence or absence of activational systems (i.e., T levels). Of course, research in other species, including reptiles and mammals, have similarly highlighted the important combination of organizational effects on reproductive behavior (Cox et al., 2017; Cox et al., 2022; Lerner and Mason, 2001; Wittman et al., 2021).

While specific kinds of organizational effects associated with behavioral diversification may depend on the species and behavior under study, there are several examples of how sex steroids modify the development of neuromotor systems used for sexual interactions. One well-known case is the dramatic neural dimorphism that underlies behavioral differences between the sexes in songbirds. In zebra finches, for example, the forebrain regions controlling song are several times larger in males, who produce learned song, than in females who do not sing (Nottebohm and Arnold, 1976). When these neural circuits are forming, steroid hormones regulate the processes of neuronal growth, survival, and synaptogenesis in the brain's song system (Arnold, 1992; Gurney, 1981), thus influencing the number of neurons and the strength of their connections in the motor pathway that generates a song's pattern. Across songbird species, it appears that sexually dimorphic singing behavior co-evolves with these steroid-mediated sex differences in the neural circuitry controlling song (Ball et al., 1994; Brenowitz, 1997; MacDougall-Shackleton and Ball, 1999). A larger sexual dimorphism in song repertoire generally means a larger difference between the size of male and female brain nuclei in a given species. Thus, it is reasonable to conclude that similar changes in the steroid-dependent developmental processes that shape the brain can yield convergent behavioral phenotypes in different species.

Other studies in songbirds show seasonal plasticity in the size of the song control nuclei, with certain nuclei increasing dramatically in the spring when individuals (males) sing prolifically (Nottebohm, 1981). These changes are in part driven by gonadal T. If, for example, males in the non-breeding season are given exogenous T, then their song control nuclei enlarge and singing activity increases (Balthazart and Ball, 2016; Schlinger and Brenowitz, 2002). Similarly, if ovariectomized females are given exogenous T, they too show a dramatic increase in the size of their song control nuclei, and they begin to sing male-like song (Nottebohm, 1980). At first, one may interpret these findings as evidence that seasonal modulation of the song system is governed largely through the activational effects of T, given that the effects appear the same in both sexes (in other words, if organizational effects were at play, then dramatic sex differences should be apparent). However, a closer look reveals that T treatment of non-breeding males and ovariectomized females generates fundamentally different results in terms of exactly how the behavior is produced (e.g., singing rate and song structure) (Barros dos Santos et al., 2022; Madison et al., 2015). Even measures of song system growth differed: the volume of forebrain song control nuclei and the rate of neurogenesis in T-treated females was still significantly smaller than in T-treated males (Barros dos Santos et al., 2022; Cornez et al., 2020). Although some of these effects may be attributed to sex differences in the brain's ability to metabolize androgens into estrogens, the effects are also attributed to differences in the mechanisms that determine neuronal growth and survival early in life through the organizational effects of sex steroids (Barros dos Santos et al., 2022).

Still, the studies described above do not address the genetic and/or molecular mechanisms by which steroidal effects on development evolve to confer behavioral variation. This process presumably involves steroids selectively acting throughout the brain and body at different time points during development to help mediate the programming of specific traits. Principles that determine what these effects might look like are articulated in the field of evolutionary developmental biology, or evo-devo (Carroll, 2005; Kirschner and Gerhart, 2008; Sanger and Rajakumar, 2019). We will not provide a full review of this field herein, as our aim is instead to point out to researchers that the dynamics between endocrinology and evo-devo can lead to a wide range of rich and interesting questions in the field of animal behavior. Hoke et al. (2019) make a similar point by arguing that developmental phenomena such as versatility, weak linkage, and exploratory mechanisms buffer phenotypic evolution by creating neurobiological robustness that allows for mutations to accumulate without disrupting behavior (or other traits). However, some developmental mechanisms exist near a “tipping point,” or natural threshold that determines alternate developmental trajectories. If a cell or tissue is pushed past one of these tipping points, then the natural process of development may generate a different phenotypic outcome. Thus, steroid modulation may act as a physiological trigger that pushes certain developmental programs past their tipping point to have an outsized effect on the way traits are (or are not) manifest. In this way, we can think of steroid hormone action as a potential mechanism to fine-tune an organism's developmental landscape, altering the molecular systems that organize how it is built.

It is not abundantly clear exactly how sex steroids would induce these effects or how differences in them would evolve. Our best guess is that selection for an altered steroid-dependent developmental program occurs through changes to molecular machinery that mediates steroidal modulation of the developmental process itself (De Robertis, 2008). This could involve slight changes to expression patterning of sex steroid receptors early in life, which would then presumably alter how these hormones influence the maturation of the nervous system and its ability to control behavior. Similarly, there are a wide range of other proteins that help mediate the effects of steroid hormone receptors on the genome, and the expression patterns of these players might also change in a functionally meaningful manner. Support for this view is scant, but there are studies that show species differences among neural expression profiles of steroid hormone receptors in neonates (Pryce, 2008; Sheng et al., 2004). Again, we suspect that by integrating the fields of behavioral endocrinology and evo-devo, researchers will gain access to an entirely new suite of questions about hormone system diversification and its contribution to behavioral diversity in the animal world.

4. Frogs as a model to study the evolution of organizational effects of steroids

Frogs are an excellent model for testing the relationships between the organizational effects of steroid hormones and behavioral evolution for two reasons. First, frog development is already a well-established model for evo-devo research on the physiological mechanisms that underlie behavioral plasticity (Ledón-Rettig and Ragsdale, 2021; Ledón-Rettig and Pfennig, 2011). Unlike other taxa, anuran development is defined by a dramatic remodeling of virtually every body system to accommodate a shift from an aquatic to a terrestrial lifestyle. Accompanying this transition is a remodeling of the frog nervous system to control behaviors that support a new diet, a new form of respiration, and a switch from tail-based swimming to limb-based locomotion. Hormones are intimately involved in directing these developmental switches, and they therefore play a key role in shaping the evolution of many different types of behaviors. For example, the primary hormones that direct metamorphosis (thyroid hormones, corticosterone, hypothalamic corticotropin releasing factor) have been linked to major evolutionary transitions in the timing of limb development and the organization of neural circuits that control limb movement (Denver, 1997; Schlosser, 2003) and to the evolution of novel feeding behaviors (Ledon-Rettig et al., 2010). In short, researchers have already identified some key organizational effects of hormones that can promote trait evolution in anurans.

Second, sex steroids in particular have a well-known role in organizing the neuromotor systems that underlie courtship behavior in frogs (Watson et al., 1993; Watson and Kelley, 1992). In *Xenopus* frogs, sexually dimorphic sensitivity to gonadal hormones during development primes neuromotor systems to support sexually dimorphic vocal behavior by modifying the anatomy and physiology of structures used in calling (Watson and Kelley, 1992). For example, in the laryngeal muscles of adult *Xenopus laevis*, the expression of AR is 3–4 times greater in developing males, compared to females (Kelley et al., 1989; Segil et al., 1987). These muscles are used to generate the sound pulses that make up the male advertisement calls, and activation of these tissues' AR mediates the specific growth and differentiation of the male larynx from an initial female-like anatomy present in both sexes (Sassoon et al., 1987; Sassoon et al., 1986). Because of this modified developmental trajectory, sex differences in the effector muscles controlling vocalizations are quite dramatic. In adulthood the male larynx has 6–7 times more muscle fibers than the female larynx (Sassoon et al., 1986), and each muscle fiber is larger in males (Gray et al., 1985). Moreover, male laryngeal muscles are made up of entirely fast twitch fibers, which are fatigue-resistant to enable the rapid (70 Hz) series of muscle contractions that produce trills (Sassoon et al., 1987; Tobias and Kelley, 1987; Tobias et al., 1991). By contrast, female laryngeal muscle fibers are mostly slow-twitch (Sassoon et al., 1987; Tobias et al., 1991), and thus female vocalizations are much simpler and slower in tempo. The organizational effects of sex steroids on the peripheral signal-generating machinery in *Xenopus* appear to be long-lasting; castration in adult males does not affect laryngeal mass or morphology, even 3 years after testes removal (Segil et al., 1987; Watson et al., 1993). These phenomena may be common to a variety of species in which androgenic effects during development primarily support the evolution of the male reproductive phenotype.

Evolutionarily, studies suggest that developmental effects of sex steroids on the larynx are likely targets of selection for diversification of vocalization behavior among *Xenopus* frogs. Male advertisement calls vary in their temporal and spectral features across the *Xenopus* phylogeny (Leininger and Kelley, 2013). For example, in *Xenopus borealis*, males have evolved a shorter, single-click call that is more like female vocalizations. In this species, the evolutionary loss of behavioral sex differences compared to other *Xenopus* species appears linked to the loss of certain aspects of sexual dimorphism in the morphology and physiology of the larynx (Leininger and Kelley, 2015; Leininger et al., 2015). In particular, *Xenopus borealis* males might have lost the androgen-dependent developmental programs that produce the masculinized fast twitch muscle fiber type (Kelley et al., 2020). This intriguing case provides an example of how novel patterns of behavior may emerge in tandem with the gain or loss of a steroid-mediated developmental program in the neuromotor system that underlies the behavior.

4.1. Development of a novel sexual display in foot-flagging frogs

Here, we introduce foot-flagging frogs as an important animal model that can help us better understand the evolution of activtional and organizational effects of sex steroids on behavior. Foot flagging is part of an elaborate display repertoire that occurs in a small number of species from across the anuran phylogeny. It is produced largely by males when they “wave” at competitors with the hindlimbs (Hödl and Amézquita, 2001). Kinematic studies reveal that this behavior is generated through a series of precise movements, in which individuals i) extend the hindlimb above the head, ii) rotate the limb downward slowly in an arch, while iii) exposing brightly colored inter-digitated foot webbing (Anderson et al., 2021a; Anderson et al., 2021b; Hödl and Amézquita, 2001). Functionally, the foot flag is used as an agonistic display to deter rivals and/or defend perching sites at breeding aggregations near waterfalls or in fast-flowing torrent streams (Preininger et al., 2013a; Preininger et al., 2009; Preininger et al., 2013b; Preininger et al., 2013c). Many speculate that the display evolved to complement or replace acoustic communication in these noisy environments (Hödl and Amézquita, 2001).

The best-studied foot-flagging frog is the Bornean rock frog, *Staurois parvus*. In this species, foot-flagging behavior in males is activated by androgens. Adult males given T increase the frequency with which they foot flag within a few hours (Mangiamele et al., 2016). T administration also changes how *S. parvus* performs foot-flag displays. When males are given T, they produce foot flags that appear more circular (Anderson et al., 2021a). Modeling work suggests that rival males can perceive these differences in foot flag shape, and thus in theory can decipher displaying males with elevated T from those with lower levels of T. Furthermore, more circular foot flags contain more vertical leg movements, with include gestures where the extended hindlimb is moved downward in a direction that is perpendicular to its long-axis. This is important, because this specific movement likely triggers an ancient feature analyzer system that originally evolved in frogs and toads to detect and classify unfamiliar environmental stimuli, like predators (Ewert, 1984, 1997). T-treatment therefore seems to mediate the production of foot flags that are more effective in exploiting this perceptual bias (Anderson et al., 2021a), possibly making the display itself appear more “dangerous” to rivals (Amézquita and Hödl, 2004; Grafe et al., 2012; Hödl and Amézquita, 2001; Preininger et al., 2013b).

Many of the effects described above likely occur through AR. When T is administered alongside an anti-androgen (e.g., flutamide), individuals greatly reduce the number of foot flags they perform (Eigerman and Mangiamele, 2022; Smith et al., 2021). Moreover, anti-androgens also disrupt the broader display routines that *S. parvus* produce, with less integration among the various signals that help mediate sexual agonistic interactions (Eigerman and Mangiamele, 2022). This result is especially important because it suggests that androgens are likely working centrally to coordinate the multiple signaling modalities (e.g., gestural display, vocalizations) that make up the frog's display repertoire, and the neural basis of these effects could certainly be organized during development.

Comparative studies suggest that the emergence of foot-flagging behavior in *S. parvus* is marked by a nearly 10-fold increase in AR expression in the limb muscles that actuate this display (Mangiamele et al., 2016). Much like in manakins, we can interpret these results in the context of the experimental studies described above, which test the effects of androgens on display output. Together, these studies support the hypothesis that selection for foot flagging likely proceeds through evolutionary increases in muscular androgenic sensitivity. Furthermore, the coupling between the evolution of foot-flagging behavior and increased muscular AR sensitivity is not specific to *S. parvus*, but rather also applies to other unrelated frogs that have convergently evolved this display (Anderson et al., 2021c). For example, muscular AR marks the evolution of foot flagging in some *Micrixalus* frogs, as well as at least one species of *Dendropsophus* tree frogs. Phylogenetic analyses that illustrate this point also suggest that correlated evolution between these two traits occurs in a mosaic fashion, where the rates and degree of change in AR levels may differ among lineages. Importantly, this work also implies that AR expression levels in the nervous system are not related to the evolution of foot flagging per se, which is consistent with the notion that correlated evolution of the traits that underlie foot-flagging behavior occurs specifically in the muscular system.

As with many of the animal systems that are used to explore the relationships between behavioral evolution and hormone system evolution, the work in foot-flagging frogs has largely been done in adult males. But, we argue that foot-flagging frogs can serve as a model for understanding how changes in both activational and organizational effects of steroid hormones may coincide with behavioral evolution. Unlike many other studies that explore this topic, sex comparisons may not be the only way to assess the impact of sex steroid hormones on the neural systems that underlie behavior; rather, *S. parvus* provides a potential opportunity to examine the ontogeny of foot-flagging behavior and its hormonal basis in a phylogenetic context. Juvenile *S. parvus* readily foot flag almost immediately after metamorphosis, which refers to when a tadpole changes into a frog. The time of metamorphosis in this species is ≈90 days after hatching from the egg; thus, we find that *S. parvus* can (and do) foot flag as soon as they “get legs” and inhabit dry land! Observational work suggests that juvenile frogs do not use foot flags as an agonistic display during bouts of competition over mates at this early life stage, but instead use the signal to compete over resources such as food (Preininger et al., 2012; D. Preininger, personal communication). Interestingly, adult males no longer seem to use the signal in this capacity.

One way to approach disentangling which components of the foot-flagging display are potentially influenced by the organizational effects of sex steroids is to conduct detailed comparisons of foot flag kinematics between adult and juvenile frogs. Here, we present such comparisons, using video recordings collected from individuals who make up a large breeding population at the Vienna Zoo where we have worked previously (Anderson et al., 2021a; Anderson et al., 2021b; Anderson et al., 2021c; Eigerman and Mangiamele, 2022; Mangiamele et al., 2016; Smith et al., 2021). Individuals here inhabit a massive enclosure that closely mimics the tropical forests of Borneo, containing a small rocky waterfall with a calm pool underneath. Adult males gather on the sides of the noisy waterfall and foot flag at each other, as they compete to gain access to females for amplexus. To obtain juveniles from this “semi-wild” population, we simply located small frogs that were within ≈3–6 months of completing their metamorphic transition from tadpole to frog (such individuals are roughly one half the size of an adult male, so they are easy to spot and observe). We then collected high-speed video recordings of their foot-flag display during contests with rivals around feeding sites (Fig. 2A; detailed methods published in: Anderson et al., 2021a; Anderson et al., 2021b. See also supplementary materials). The video recordings of displaying adult males that we used as a comparison are from previous work, in which we established how to quantify and compare foot flag shape from free-living individuals (Anderson et al., 2021b).

As with many displays that can be compared across life stages, we expected that the geometry of juvenile foot flags would differ from that of adult males. In particular, we anticipated that the vertical component of the display, which likely depends on AR activation within the hindlimb motor system (see above), is where we should see the most striking differences, such that adult males would generate more vertical movement in their foot flags than juveniles. We also recognize, however, that juveniles are much smaller than adult males; therefore, one might expect that differences in foot flag shape could be attributed solely to ontogenetic variation in body size. If so, then we would expect all components of the foot flag to differ between juveniles and adult males.

Overall, our results were consistent with the idea that foot-flagging behavior changes as individuals mature. For example, we found that foot flag shape differed between juveniles and adult males, although the effect itself was marginal (Wilk's lambda = 0.098, $F_{4,8} = 4.59$, $p = 0.079$, Fig. 2B). This trend was mostly attributed to statistically significant differences in arch component of the display, where the male slowly lowers his foot down to the substrate with his interdigitated foot webbing fully exposed and visible to his rival. Juveniles produce smaller arch movements compared to adult males ($t_{10.75} = 2.421$, $p = 0.034$, Fig. 2C), and this effect is seen both in terms of the arch movements' vertical ($t_{11.55} = 2.33$, $p = 0.039$, Fig. 2E) and horizontal ($t_{10.98} = 2.22$, $p = 0.048$, Fig. 2D) spatial components. The only other major difference in the foot flags of juvenile frogs was the lowering distance, or the distance the leg is dropped to the substrate after the display is completed, which was shorter in juveniles than in adults ($t_{11.19} = 3.00$, $p = 0.012$, Fig. 3A). All other components of the foot flag were statistically indistinguishable between juveniles and adults, including the leg lift ($t_{3.933} = 0.38$, $p = 0.72$), extended knee ($t_{4.321} = 0.47$, $p = 0.67$), and pull-in ($t_{3.285} = 0.018$, $p = 0.99$). Altogether, these results highlight select differences in the foot flag between juvenile and adult individuals. Our findings are also consistent with the notion that such differences are not exclusively due to differences in body size between juveniles and adults, as only certain parts of the foot flag are in fact different.

Other aspects of our analysis include an exploration of path length (distance the foot travels) and duration (time of foot movement). We found that juveniles produce a foot flag with a significantly shorter total path length ($t_{8.14} = 2.70$, $p = 0.026$, Fig. 3B). This means that juveniles likely move their foot over a shorter distance than adults, likely because of the reduction in arch phase. We also found that duration of the foot-flag display is much shorter in juveniles compared to adult males ($t_{11.82} = 4.81$, $p < 0.001$, Fig. 4C). On average, adult males have slower extended knee ($t_{9.28} = 3.23$, $p = 0.010$, Fig. 4A) and arch ($t_{11.19} = 3.74$, $p = 0.003$, Fig. 4B) components of the foot flag. These analyses therefore support the idea that the distance and speed with which a male moves its foot to generate a display varies ontogenetically, such that adults tend to increase the path of movement and slow the speed of the foot (or some combination of the two).

Our data also suggest that adult males show seemingly higher levels of variation in their foot-flag displays than juveniles (see Figs. 2–4). We can clearly see that some adult males perform slow displays with large arch phases, whereas other adult males perform faster displays with smaller arch phases. Juveniles, on the other hand, tend to cluster in terms of the parameters that describe the shape their foot-flag display, generating displays that appear more as an upward leg extension without much of an arch. We suspect that these differences are related to the extent to which the motor control systems of the limb are refined to produce the precise movements that underlie the agonistic display of sexually mature adult males (Anderson et al., 2021a). This view is also consistent with our work suggesting that adult foot flags are dynamic, in

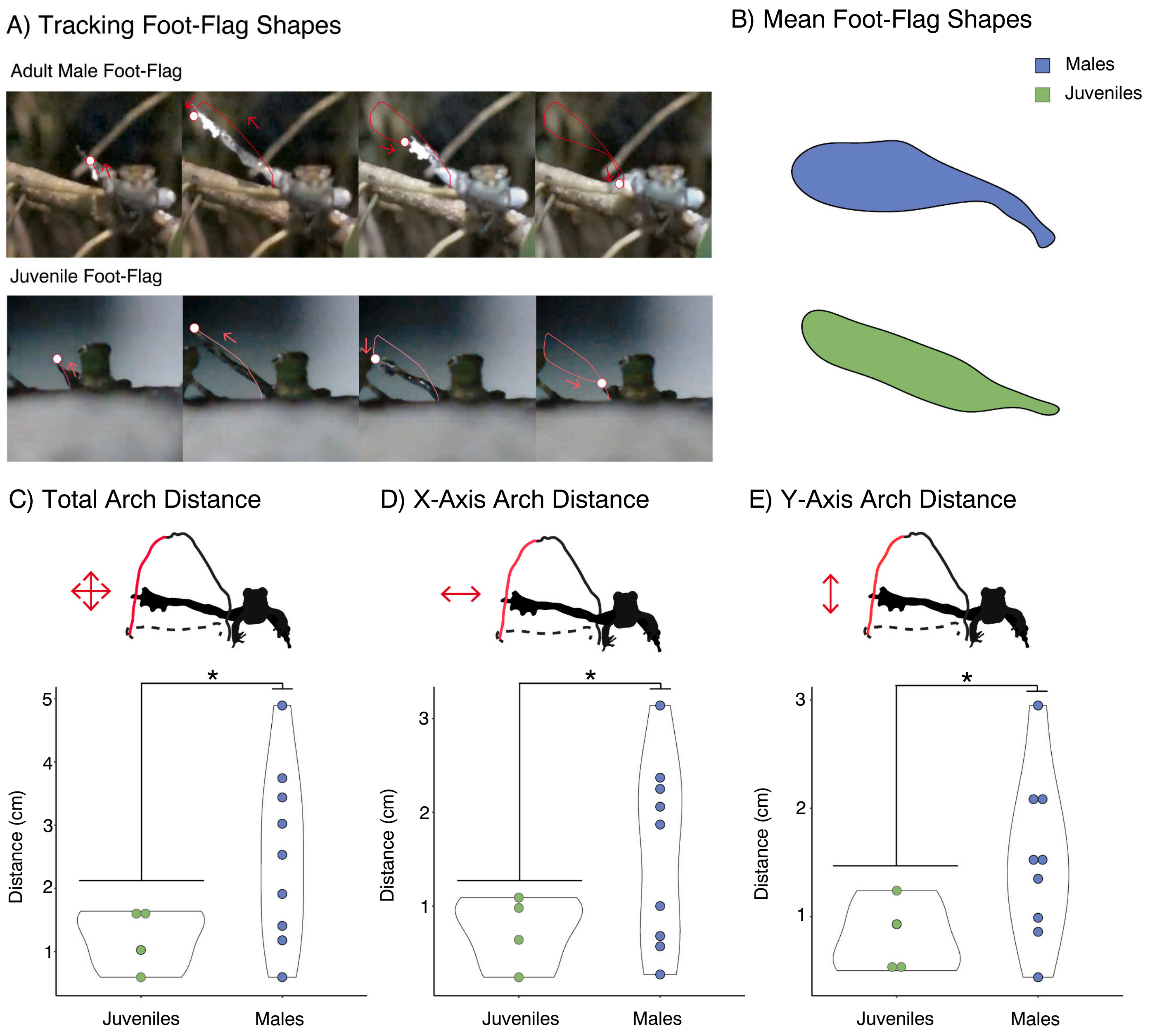

Fig. 2. We collected high-speed video recordings of foot-flagging behavior for males and juveniles with a Sony RX 10 II slow motion camera at 250 fps (see supplemental methods for further details). (A) Demonstration of foot tracking using Kinovea software. Red line represents the trace of the foot as it moves through the air, while the white dot is the tracking point on the middle toe. The red arrows represent directionality of the foot movement. (B) Average foot-flag shape for adult (blue) and juvenile (green) males. (C) Violin plot showing the total arch displacement in centimeters (cm) of juveniles and adult males. (D) Violin plot showing the x-axis displacement of the arch component in cm of juveniles and adult males. (E) Violin plot showing the y-axis displacement of the arch component in cm of juveniles and adult males. Asterisks (*) signify significant comparisons ($p < 0.05$).

that their shape might be influenced substantially by levels of circulating androgens.

Building on both our previous research and our preliminary findings in juveniles described above, we can leverage these current results to shed a light on activational vs. organizational effects of T on the foot flag. Specifically, we were interested to examine whether ontogenetic differences in the foot-flag display – namely the arch phase – are attributed to differences in the androgenic system. Thus, we collected free-roaming juveniles and adult males from the breeding population at the zoo. Similar to past studies (Anderson et al., 2021a; Mangiamele et al., 2016), we then euthanized these individuals and dissected their hind limbs muscles (see Supplemental Methods for further methodological details). During the dissection, we verified that all individuals were in fact males by inspecting their gonads. We then used quantitative PCR to measure relative AR expression in the muscle tissue (Anderson et al., 2021c; Mangiamele et al., 2016) and tested whether juvenile hindlimb muscles express lower levels of AR transcripts than adult male hindlimb muscles. Indeed, our results supported this hypothesis, as adult males maintained relatively higher levels of AR mRNA in their hindlimb leg muscles than juveniles ($t_{5.18} = -2.055$, $p = 0.047$, Fig. 5). This result suggests that muscular AR increases sometime during post-metamorphic development. We further hypothesize that this ontogenetic shift in androgen sensitivity likely contributes to the differences in androgenic regulation of the foot-flag display, particularly those differences related to control of vertical leg movements.

At the same time, our results clearly show that juvenile frogs are fully

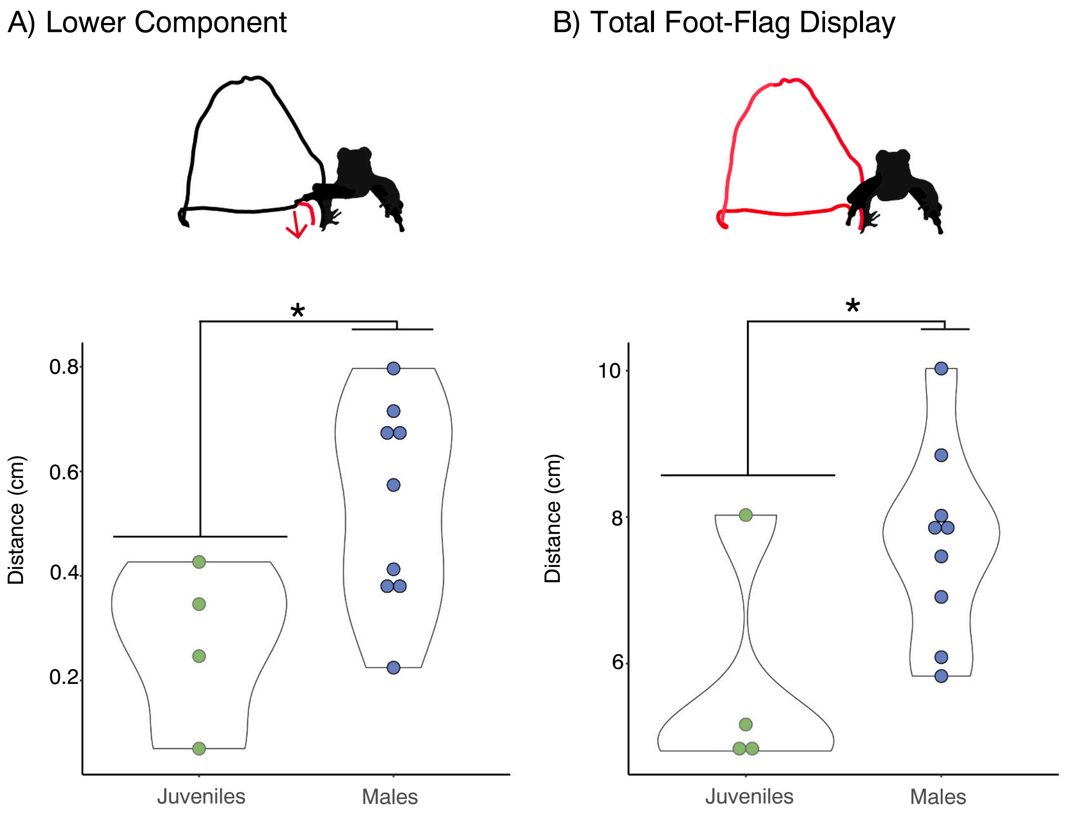

Fig. 3. (A) Violin plot showing the total lower component displacement in centimeters (cm) of juvenile and adult males. (B) Violin showing the total foot-flag display distance of juvenile and adult males. Asterisks (*) signify significant comparisons ($p < 0.05$).

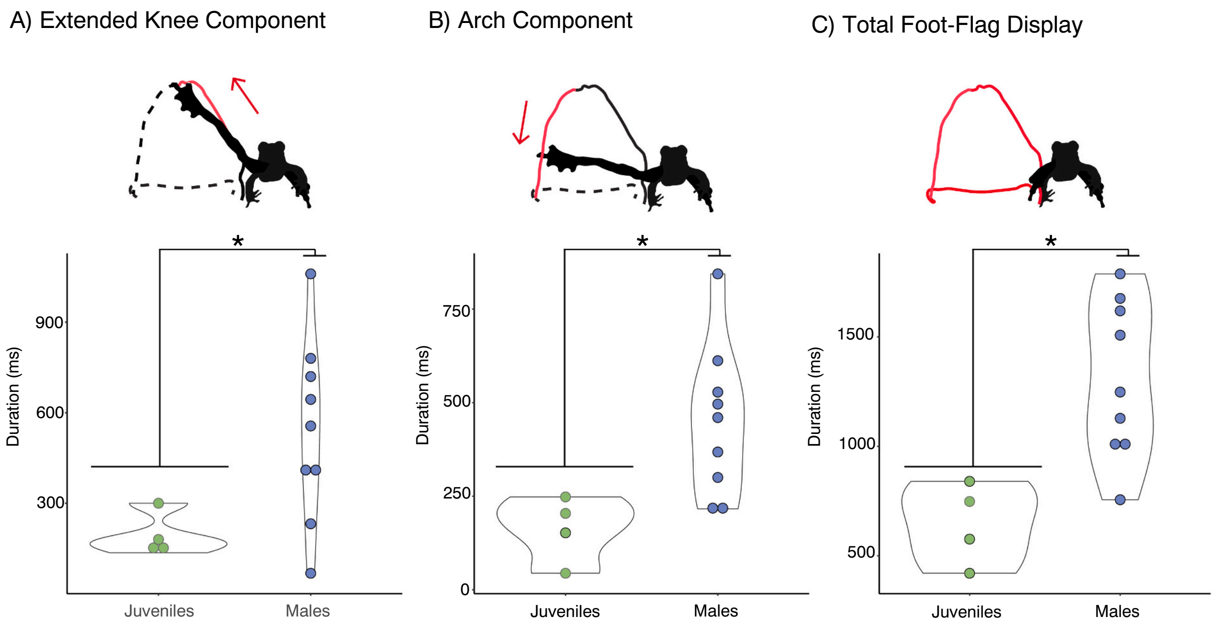

Fig. 4. We compared duration of the foot-flag display and its different components between juveniles and adult males using the x- and y-coordinates of the foot flag trajectories using Welch's two sample *t*-tests. (A) Violin plot showing the duration of the extended knee component of the foot flag in milliseconds (ms) of juvenile and adult males. (B) Violin plot showing the duration of the arch component of the foot flag in ms of juvenile and adult males. (C) Violin plot showing the duration of the total foot-flag display in ms of juvenile and adult males. Asterisks (*) signify significant comparisons ($p < 0.05$).

capable of generating a foot-flag display, regardless of differences in the androgenic system (at least differences we know about). This means that the basic motor program necessary to perform this signal is likely in place at the time of metamorphosis, and thus there are undoubtedly organizational processes that help lay down the neural circuits for this behavior. We suspect that some of these effects can again be observed by comparing the neural systems of juvenile and adult frogs, as differences may provide clues about the neurobiological traits that help determine

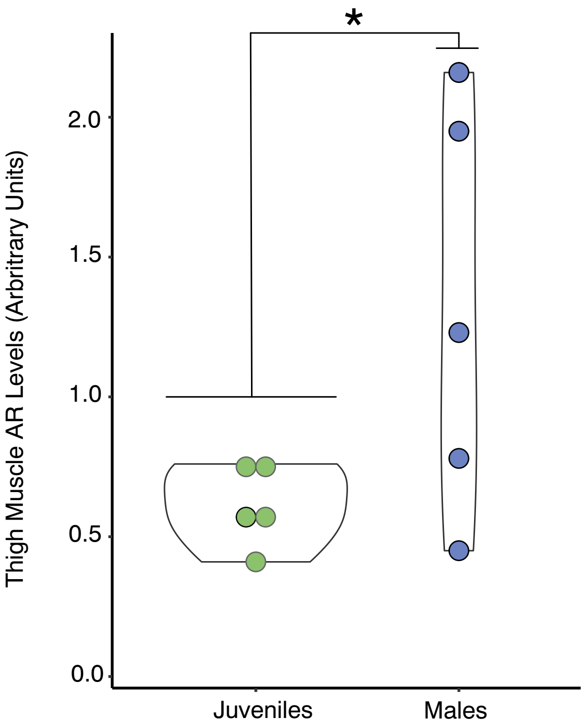

Fig. 5. Violin plot of relative androgen receptor (AR) transcript levels in the leg muscles of reproductively active adult males ($n = 5$) and juveniles ($n = 5$) of *Staurois parvus*. Asterisk (*) signifies significant comparison ($p < 0.05$).

the kinematics of a foot flag.

We have begun some preliminary comparisons of the spinal cord in juvenile and adult *S. parvus* by measuring basic morphological features of cells that coordinate and actuate movement. Specifically, we measured the density and diameter of motoneurons and their surrounding interneurons in the lateral motor column of the lumbar spinal cord, which control the hind limb. To date, we have uncovered several anatomical differences that accompany the observed kinematic changes in foot-flagging behavior as the frogs mature. We found a much higher density of motoneurons in adults compared to juveniles (GLM, $X_1^2 = 21.268$, $p < 0.001$, Fig. 6A). Interneuron density trended in the same direction as well (GLM, $X_1^2 = 3.0326$, $p = 0.08$, Fig. 6B). In terms of cell size, interneuron diameters were significantly larger in the adult compared to the juvenile spinal cord (LME, $F_1 = 4.98$, $p = 0.046$, Fig. 6D). Motoneuron diameters also trended in this direction, with the results approaching significance (LME, $F_1 = 22.49$, $p = 0.07$, Fig. 6C). These results demonstrate that cells in the spinal cord increase in size and proliferate as foot-flagging frogs grow. Such growth might be expected; in frogs, the larger the target muscle mass, the more motoneurons that are needed to control it (Farel et al., 1993). Yet, this developmental trajectory may also suggest that increasing AR activation could play an important role in the organization of the spinal motor circuits that control foot flag production. We know that in other species androgens support many morphological changes in the spinal cord (Forger and Breedlove, 1987; Kurz et al., 1986) – including increases in motoneuron soma size (Fraley and Ulibarri, 2002) – that can improve fine control over the innervated muscles, enhance motor output, and increase agility (Song et al., 2016). Therefore, if the organizational effects of androgens encompass changes in neuron size and density in the spinal cord of *S. parvus*, that might explain the observed changes in foot flag performance with age. For instance, we would expect ontogenetic changes in spinal interneurons to influence hind limb movement kinematics and their underlying muscle synergies (d'Avella et al., 2003; Saltiel et al., 2001). Together, these results suggest that although the neuromotor circuitry required to generate a foot flag is present even at the early post-metamorphic stage, developmental changes in neural morphology coincide with developmental changes in behavior that are important for signaling.

With these data in mind, we argue that foot-flagging frogs offer us a rare chance to investigate how evolutionary changes in the organizational effects of hormones can impact the expression of behavior. This is because there is a rich body of literature in frogs dating back to the 1960s that provides us with an in-depth understanding of the development of the neural circuitry that underlies hindlimb motor behavior. We understand how hindlimb motoneurons proliferate, grow, and differentiate, when during development hindlimb locomotor circuits become functional, and what patterns of neural activity and hindlimb behavior look like at each stage of leg growth (Farel, 1987; Sperry and Grobstein, 1985; Stehouwer and Farel, 1984, 1985). It would therefore be feasible to characterize the effects of hormonal manipulations on these established developmental trajectories, or to identify patterns of diversification and convergence in the nervous systems of frog species that do and do not foot flag. Thus, in foot-flagging frogs, we have the opportunity to study the influence of sex steroid hormones on the development of hindlimb behavior from the first emergence of limbs to their use in adult sexual displays and to compare these effects across species to reveal signatures of evolutionary change.

5. Evolution of the organizational and activational systems that underlie foot-flagging behavior

How can we tease apart the evolution of activational and organizational effects to better understand their impact on the emergence of foot-flagging behavior? As we describe above, there are clear ontogenetic differences in the nervous system that are associated with the size and shape of the foot-flag display, but not necessarily the presence or absence of this signal. Moreover, we do not think that high circulating T is necessary to produce the foot flag, given that we have recorded it (see above) in juvenile males that have only recently completed their metamorphic transition from the tadpole stage. Juveniles likely have less circulating T than adult males (e.g., in *Xenopus*, circulating T levels in late stage juveniles is approximately half that of sexually mature adult males; Kang et al., 1995), and we show above that they also have lower expression of muscular AR in their hindlimb. Therefore, we suspect that AR in the hindlimb muscles increases later in life, presumably when males become reproductively mature. Altogether, these data imply that there is likely a strong neuroendocrine motor phenotype that is linked to the foot-flag display, with some of this phenotype arising during the developmental process.

At a mechanistic level, there are a range of ways that organization of the foot flag might unfold (Fig. 1; see also framework outlined by Katz and Hale, 2017). First, selection may act on the systems that govern how sex steroids organize the development of the anatomical "wiring" that underlies motor control of the hindlimb (Fig. 1B). Such effects likely occur during tadpole development and/or early metamorphosis, which is when neural circuits controlling the limbs are first laid down. In particular, we might predict that AR influences the developmental trajectory of spinal motor circuits by altering a developmentally programmed period of cell death that begins when limb buds first appear (Farel et al., 1993; Pollack, 1988). Such a mechanism of hormonally-mediated developmental reprogramming of limb neural circuitry in frog larvae is already known to be associated with the evolution of direct development, in which thyroid hormone influences the survival of neurons in the lateral motor column to provide early control over movement of the limbs in frogs that never go through a tadpole stage (Schlosser, 2003).

Expanding on the ideas described above, we hypothesize that AR influences neurodevelopment in a manner that provides better control over limb movement. One way to exert such an effect is to increase the number of cells in the spinal cord that connect to hind limb muscles. In fact, juvenile foot-flagging frogs tend to have fewer motoneurons and

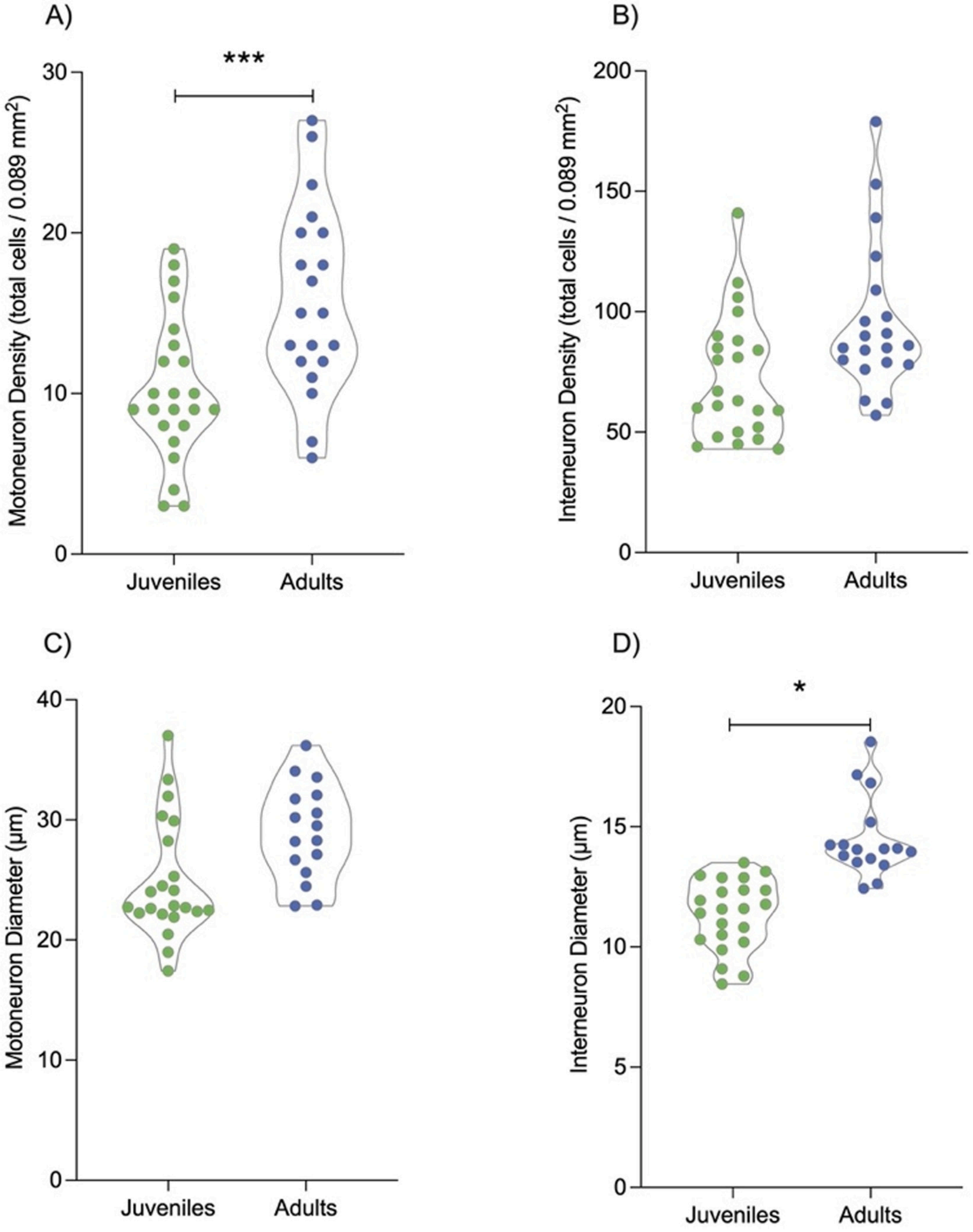

Fig. 6. Comparison of motoneurons and interneurons in juvenile and adult foot-flagging frogs (*S. parvus*). (A) Motoneurons density, (B) interneuron density, (C) motoneuron diameter, (D) interneuron diameter. Asterisks (*) signify significant comparisons ($p < 0.05$).

interneurons in the lumbar spinal cord compared to adults (Fig. 6). These neurons also appear to be smaller in juveniles (Fig. 6). AR might therefore act during development to increase the number and size of spinal motor- and interneurons. How this occurs is not precisely known, but it would likely involve AR-dependent regulation of trophic factors that influence cell shape, size, etc. (Hiipakka and Liao, 1998; Massie et al., 2007; Quartier et al., 2018).

Alternatively, organizational effects of AR that are necessary for foot flagging might occur outside of the nervous system. Muscle is a major androgen target, and thus T might work via AR to modify muscle structure and/or function as a means of supporting foot flag performance. If so, then we might predict developmental changes in AR density in the muscle, but maybe not in the central nervous system. Juvenile *S. parvus* do indeed have lower levels of AR in their hindlimb musculature compared to adult males (Fig. 5). If evolutionary changes that occurred in the muscle are critical to performing the foot-flag display, we might also predict that, like in the manakin bird (Fuxjager et al., 2013), blocking AR at the periphery is sufficient to disrupt foot-flagging behavior, either in juveniles or adults. One caveat is that we do not yet know whether foot-flagging frog hindlimb muscles have any unique features compared to the same muscles in non-foot-flagging relatives, nor whether their structure or function changes during the developmental transition from juvenile to adult. Blocking ARs during developmental transitions and measuring muscle morphological features, such as fiber type or number, or physiological properties, such as twitch speed, would help us determine whether such changes have evolved in tandem with changes in hindlimb androgen sensitivity.

Androgenic action via AR in muscle during development may also influence the nervous system indirectly. There is a large body of work in rodents that shows that AR action within the peripheral muscular system initiates the expression of trophic factors, which retrogradely travel via the motoneuron to the spinal cord (Rand and Breedlove, 1995; Verhovshek and Sengelaub, 2013). There, the trophic factors initiate a host of morphological changes to the cell that can influence motor skills and ability (Kaspar et al., 2003; Song et al., 2016). These effects are also likely important during embryonic development (Connor and Smith, 1994; Ladle et al., 2007; Li et al., 2008); thus, at least, it seems plausible that AR action at the level of the muscle during the development similarly helps shape the circuitry in the spinal cord that is necessary to generate foot flags.

With these considerations in mind, we must recognize that selection of organizational effects of sex steroid on the nervous or muscular systems are potentially balanced with selection on the activational effects of sex steroids (Fig. 1A). How this balance occurs in the context of evolution remains a mystery, and this topic may even be one of the biggest puzzles of evolutionary endocrinology. In any case, the answer may be uncovered by studying how exactly androgens (and possibly other sex steroids) mediate neural and muscular function to change the probability of foot-flagging behavior. For example, high androgen levels could act to change the synaptic properties of the motoneurons, releasing more neurotransmitter when AR is activated (Wu et al., 2001), and/or androgens could affect the physiology and performance of hind limb muscles (Regnier and Herrera, 1993). We see evidence of activational effects of androgens on foot flagging in many ways (see above), yet future work will have to explore (as in manakins) how much of these effects are owed to sex steroid action during development and how selection has altered this process to help give rise to circuitry that facilitates the foot-flag display.

6. Summary

Here, we explore the long-acknowledged effects of sex steroids on the activation and organization of vertebrate reproductive behavior. In doing so, we point out that when selection drives the evolution of difference in reproductive behaviors among taxa, it likely acts on these mechanisms. This means that the diversification of sex steroid regulatory mechanisms presumably occurs through processes that govern not only the development of key neural systems to control behavior, but also the way these systems are activated later in life during adulthood. Less is understood about the former, creating a major gap in the way we understand hormone-behavior relationships and their role in behavioral variation across the animal tree of life.

We also introduce the foot-flagging frog as an intriguing animal model to study the evolution of organizational effects through sex steroid action. Like in most frog species, individuals undergo a metamorphic transition when they transform from a tadpole into their “terrestrial form” (although, not all frogs are terrestrial). This process involves many developmental changes, including growing legs; thus, we can study in detail how the neuroendocrine systems for foot flagging are laid down when a tadpole develops its appendages. Accordingly, we present results that suggest that juvenile frogs are capable of foot flagging and, compared to adults, they maintain core differences in the spinal neuroendocrine phenotype that is associated with this behavior. We fully recognize that our results raise more questions than they answer, but nonetheless our point is merely to show the incredible potential of this relatively unexplored area of evolutionary behavioral neuroendocrinology.

Going forward, there is much work to be done. Just as researchers have begun to map out behavioral differences and their endocrine correlates on to phylogenies to better delineate how such traits might have evolved, they should also begin such work with respect to developmental endocrinology. These studies will be challenging, and they will require finding novel systems like foot-flagging frogs in which manipulation of developmental processes can most easily be achieved. Other work will need to explore how individual variation in the organizational effects of sex steroids might shape behavioral variation later in life. Such work will be vital to understanding on what developmental traits selection can act to shape an organism's adaptive behavioral repertoire. Similarly, there are still several outstanding questions related to the *how* sex steroids mediate the organization of behavior, particularly relating to the more exotic or unusual reproductive behaviors that have evolved in the natural world. Many of these traits are highly complex, and thus tap into a wide range of neurological processes that control facets of learning and memory, sensory processing, arousal, motor control, etc. We can better conceptualize this point by thinking back to the iconic examples we described in the introduction, including the ability of bower birds to construct awesome bowers as part of their courtship rituals, or the ability of *Anolis* lizards to perform elaborate head-bobbing and push-up displays for competition. How are the neural systems of these behaviors organized, and how did selection shape these organizational processes to give us this diversity? Answering questions like these represents a new frontier in endocrinology and organismal biology.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.yhbeh.2022.105248.

References

Adkins-Regan, E., 1983. In: Sex Steroids and the Differentiation and Activation of Avian Reproductive Behaviour, Hormones and Behaviour in Higher Vertebrates. Springer, pp. 218–228.

Adkins-Regan, E., 2005. Hormones and Animal Social Behavior. Princeton University Press, Princeton.

Adkins-Regan, E., 2008. Do hormonal control systems produce evolutionary inertia? Philos. Trans. R. Soc. Lond. Ser. B Biol. Sci. 363, 1599–1609.

Adkins-Regan, E., 2012. Hormonal organization and activation: evolutionary implications and questions. Gen. Comp. Endocrinol. 176, 279–285.

Amézquita, A., Hödl, W., 2004. How, when, and where to perform visual displays? The case of the Amazonian frog Hyla parviceps. Herpetologica 60, 20–29.

Anderson, N.K., Grabner, M., Mangiamele, L.A., Preininger, D., Fuxjager, M.J., 2021a. Testosterone amplifies the negative valence of an agonistic gestural display by exploiting receiver perceptual bias. Proc. R. Soc. B 288, 20211848.

Anderson, N.K., Gururaja, K.V., Mangiamele, L.A., Netoskie, E.C., Smith, S., Fuxjager, M. J., Preininger, D., 2021b. Insight into the evolution of anuran foot flag displays: a comparative study of color and kinematics. Ichthyol.Herpetol. 109, 1047–1059.

Anderson, N.K., Schuppe, E.R., Gururaja, K.V., Mangiamele, L.A., Cusi Martinez, J.C., Priti, H., von May, R., Preininger, D., Fuxjager, M.J., 2021c. A common endocrine signature marks the convergent evolution of an elaborate dance display in frogs. Am. Nat. 198, 522–539.

Anholt, R.R., O'Grady, P., Wolfner, M.F., Harbison, S.T., 2020. Evolution of reproductive behavior. Genetics 214, 49–73.

Arnold, A.P., 1992. Developmental plasticity in neural circuits controlling birdsong: sexual differentiation and the neural basis of learning. J. Neurobiol. 23, 1506–1528.

Arnold, A.P., 2009a. Mouse models for evaluating sex chromosome effects that cause sex differences in non-gonadal tissues. J. Neuroendocrinol. 21, 377–386.

Arnold, A.P., 2009b. The organizational–activational hypothesis as the foundation for a unified theory of sexual differentiation of all mammalian tissues. Horm. Behav. 55, 570–578.

Arnold, A.P., Breedlove, S.M., 1985. Organizational and activational effects of sex steroids on brain and behavior: a reanalysis. Horm. Behav. 19, 469–498.

Arnold, A.P., Chen, X., 2009. What does the “four core genotypes” mouse model tell us about sex differences in the brain and other tissues? Front. Neuroendocrinol. 30, 1–9.

Auger, A.P., 2004. Steroid receptor control of reproductive behavior. Horm. Behav. 45, 168–172.

Bailey, N.W., Gray, B., Zuk, M., 2010. Acoustic experience shapes alternative mating tactics and reproductive investment in male field crickets. Curr. Biol. 20, 845–849.

Ball, G.F., Balthazart, J., 2008. Individual variation and the endocrine regulation of behavioral and physiology in birds: a cellular/molecular perspective. Philos. Trans. R. Soc. Lond. Ser. B Biol. Sci. 363, 1699–1710.

Ball, G.F., Casto, J.M., Bernard, D.J., 1994. Sex differences in the volume of avian song control nuclei: comparative studies and the issue of brain nucleus delineation. Psychoneuroendocrinology 19, 485–504.

Balthazart, J., Ball, G.F., 2016. Endocrine and social regulation of adult neurogenesis in songbirds. Front. Neuroendocrinol. 41, 3–22.

Barros dos Santos, E., Ball, G.F., Cornil, C.A., Balthazart, J., 2022. Treatment with androgens plus estrogens cannot reverse sex differences in song and the song control nuclei in adult canaries. Horm. Behav. 143, 105197.

Bass, A.H., Chagnaud, B., 2012. Shared developmental and evolutionary origins of neural basis of vocal-acoustic and pectoral-gestural signaling. Proc. Natl. Acad. Sci. U. S. A. 109, 10677–10684.

Beatty, W.W., 1979. Gonadal hormones and sex differences in nonreproductive behaviors in rodents: organizational and activational influences. Horm. Behav. 12, 112–163.

Bentz, A.B., Dossey, E.K., Rosvall, K.A., 2019. Tissue-specific gene regulation corresponds with seasonal plasticity in female testosterone. Gen. Comp. Endocrinol. 270, 26–34.

Borgia, G., 1995. Complex male display and female choice in the spotted bowerbird: specialized functions for different bower decorations. Anim. Behav. 49, 1291–1301.

Bowyer, R.T., McCullough, D.R., Rachlow, J.L., Ciuti, S., Whiting, J.C., 2020. Evolution of ungulate mating systems: integrating social and environmental factors. Ecol.Evol. 10, 5160–5178.

Brenowitz, E.A., 1997. Comparative approaches to the avian song system. J. Neurobiol. 33, 517–531.

Canoine, V., Fusani, L., Schlinger, B., Hau, M., 2007. Low sex steroids, high steroid receptors: increasing the sensitivity of the nonreproductive brain. Dev.Neurobiol. 67, 57–67.

Carroll, S.B., 2005. Endless Forms Most Beautiful: The New Science of Evo Devo and the Making of the Animal Kingdom. WW Norton & Company.

Chiver, I., Schlinger, B.A., 2017a. Clearing up the court: sex and the endocrine basis of display-court manipulation. Anim. Behav. 131, 115–121.

Chiver, I., Schlinger, B.A., 2017b. Sex differences in androgen activation of complex courtship behaviour. Anim. Behav. 124, 109–117.

Chiver, I., Schlinger, B.A., 2019. Sex-specific effects of testosterone on vocal output in a tropical suboscine bird. Anim. Behav. 148, 105–112.

Connor, E.A., Smith, M.A., 1994. Retrograde signaling in the formation and maintenance of the neuromuscular junction. J. Neurobiol. 25, 722–739.

Cornez, G., Shevchouk, O.T., Ghorbanpoor, S., Ball, G.F., Cornil, C.A., Balthazart, J., 2020. Testosterone stimulates perineuronal nets development around parvalbumin cells in the adult canary brain in parallel with song crystallization. Horm. Behav. 119, 104643.

Cox, R.M., 2020. Sex steroids as mediators of phenotypic integration, genetic correlations, and evolutionary transitions. Mol. Cell. Endocrinol. 502, 110668.

Cox, R.M., Cox, C.L., McGlothlin, J.W., Card, D.C., Andrew, A.L., Castoe, T.A., 2017. Hormonally mediated increases in sex-biased gene expression accompany the breakdown of between-sex genetic correlations in a sexually dimorphic lizard. Am. Nat. 189, 315–332.

Cox, R.M., Hale, M.D., Wittman, T.N., Robinson, C.D., Cox, C.L., 2022. Evolution of hormone-phenotype couplings and hormone-genome interactions. Horm. Behav. 144, 105216.

Crews, D., Moore, M.C., 1986. Evolution of mechanisms controlling mating behavior. Science 231, 121–125.

d'Avella, A., Saltiel, P., Bizzi, E., 2003. Combinations of muscle synergies in the construction of a natural motor behavior. Nat. Neurosci. 6, 300–308.

De Robertis, E., 2008. Evo-devo: variations on ancestral themes. Cell 132, 185–195.

De Vries, G.J., Rissman, E.F., Simerly, R.B., Yang, L.-Y., Scordalakes, E.M., Auger, C.J., Swain, A., Lovell-Badge, R., Burgoyne, P.S., Arnold, A.P., 2002. A model system for study of sex chromosome effects on sexually dimorphic neural and behavioral traits. J. Neurosci. 22, 9005–9014.

Demas, G., Cooper, M., Albers, H., Soma, K., 2007. Novel mechanisms underlying neuroendocrine regulation of aggression: a synthesis of rodent, avian, and primate studies. In: Handbook of Neurochemistry and Molecular Neurobiology: Behavioral Neurochemistry, Neuroendocrinology and Molecular Neurobiology, pp. 337–372.

Denver, R.J., 1997. Proximate mechanisms of phenotypic plasticity in amphibian metamorphosis. Am. Zool. 37, 172–184.

Eigerman, A.R., Mangiamele, L.A., 2022. Mechanisms of multimodality: androgenic hormones and adaptive flexibility in multimodal displays. Anim. Behav. 184, 149–156.

Elbrecht, A., Smith, R.G., 1992. Aromatase enzyme activity and sex determination in chickens. Science 255, 467–470.

Emlen, D.J., Warren, I.A., Johns, A., Dworkin, I., Lavine, L.C., 2012. A mechanism of extreme growth and reliable signaling in sexually selected ornaments and weapons. Science 337, 860–864.

Ewert, J.P., 1984. Tectal Mechanism That Underlie Prey-Catching and Avoidance Behaviors in Toads. Plenum Press, New York, NY.

Ewert, J.P., 1997. Neural correlates of key stimulus and releasing mechanism: a case study and two concepts. Trends Neurosci. 20, 332–339.

Farel, P.B., 1987. Motoneuron number in the lumbar lateral motor column of larval and adult bullfrogs. J. Comp. Neurol. 261, 266–276.

Farel, P.B., Wray, S.E., Meeker, M.L., 1993. Size-related increase in motoneuron number: evidence for late differentiation. Dev. Brain Res. 71, 169–179.

Fink, S., Excoffier, L., Heckel, G., 2006. Mammalian monogamy is not controlled by a single gene. Proc. Natl. Acad. Sci. 103, 10956–10960.

Fischer, E.K., O'Connell, L.A., 2017. Modification of feeding circuits in the evolution of social behavior. J. Exp. Biol. 220, 92–102.

Forger, N.G., Breedlove, S.M., 1987. Seasonal variation in mammalian striated muscle mass and motoneuron morphology. J. Neurobiol. 18, 155–165.

Fraley, G.S., Ulibarri, C.M., 2002. Long-term castration effects motoneuron size but not number in the spinal nucleus of the bulbocavernosus in the adult male mongolian gerbil. Brain Res. 953, 265–271.

Fusani, L., Day, L.B., Canoine, V., Reinemann, D., Hernandez, E., Schlinger, B.A., 2007. Androgen and the elaborate courtship behavior of a tropical lekking bird. Horm. Behav. 51, 62–68.

Fuxjager, M.J., Barske, J., Du, S., Day, L.B., Schlinger, B.A., 2012. Androgens regulate gene expression in avian skeletal muscles. PLoS One 7, e51482.

Fuxjager, M.J., Eaton, J., Lindsay, W.R., Salwiczek, L.H., Rensel, M.A., Barske, J., Sorenson, L., Day, L.B., Schlinger, B.A., 2015. Evolutionary patterns of adaptive acrobatics and physical performance predict expression profiles of androgen receptor - but not oestrogen receptor - in the forlimb musculature. Funct. Ecol. 29, 1197–1208.

Fuxjager, M.J., Forbes-Lorman, R.M., Coss, D.J., Auger, C.J., Auger, A.P., Marler, C.A., 2010. Winning territorial disputes selectively enhances androgen sensitivity in neural pathways related to motivation and social aggression. Proc. Natl Acad. Sci. USA 107, 12393–12398.

Fuxjager, M.J., Lee, J., Chan, T., Bahn, J., Chew, J., Xiao, X., Schlinger, B.A., 2016a. Hormones, genes and athleticism: effect of androgens on the avian muscular transcriptome. Mol. Endocrinol. 30, 254–271.

Fuxjager, M.J., Longpre, K.M., Chew, J.G., Fusani, L., Schlinger, B.A., 2013. Peripheral androgen receptors sustain the acrobatics and fine motor skill of elaborate male courtship. Endocrinology 154, 3168–3177.

Fuxjager, M.J., Miles, M.C., Goller, F., Petersen, J., Yancey, J., 2017. Androgens support male acrobatic courtship behavior by enahnce muscle speed and easing the severity of its trade-off with force. Endocrinology 158, 4038–4046.

Fuxjager, M.J., Miles, M.C., Schlinger, B.A., 2018. Evolution of the androgen-induced male phenotype. J. Comp. Physiol. A Neuroethol. Sens. Neural Behav. Physiol. 204.

Fuxjager, M.J., Montgomery, J.L., Marler, C.A., 2011. Species differences in the winner effect dissappear in response to post-victory testosterone manipulations. Proc. R. Soc. Lond. B Biol. Sci. 278, 3497–3503.

Fuxjager, M.J., Oyegbile, T.O., Marler, C.A., 2011b. Independent and additive contributions of postvictory testosterone and social experience to the evelopment of the winner effect. Endocrinology 152, 3422–3429.

Fuxjager, M.J., Schlinger, B.A., 2015. Perspectives on the evoltuion of animal dancing: a case study in manakins. Curr. Opin. Behav. Sci. 6, 7–12.

Fuxjager, M.J., Schuppe, E.R., 2018. Androgenic signaling systems and their role in behavioral evolution. J. Steroid Biochem. Mol. Biol. 184, 47–56.

Fuxjager, M.J., Schuppe, E.R., Hoang, J., Chew, J., Shah, M., Schlinger, B.A., 2016b. Expression of 5α- and 5β-reductase in spinal cord and muscle of birds with different courtship repertoires. Front. Zool. 13, 25.

Garrick, L.D., Lang, J.W., 1977. Social signals and behaviors of adult alligators and crocodiles. Am. Zool. 17, 225–239.

Gatewood, J.D., Wills, A., Shetty, S., Xu, J., Arnold, A.P., Burgoyne, P.S., Rissman, E.F., 2006. Sex chromosome complement and gonadal sex influence aggressive and parental behaviors in mice. J. Neurosci. 26, 2335–2342.

Ginsberg, J., Rubenstein, D.I., 1990. Sperm competition and variation in zebra mating behavior. Behav. Ecol. Sociobiol. 26, 427–434.

Gioiosa, L., Chen, X., Watkins, R., Klanfer, N., Bryant, C.D., Evans, C.J., Arnold, A.P., 2008. Sex chromosome complement affects nociception in tests of acute and chronic exposure to morphine in mice. Horm. Behav. 53, 124–130.

Goy, R.W., Phoenix, C.H., 1972. The effects of testosterone proprionate administration before birth on the development of behavior in genetic female rhesus monkeys. In: Sawyer, C., Gorski, R. (Eds.), Steroid Hormones and Brain Function. University of California Press, Berkeley.

Grafe, T.U., Preininger, D., Sztatecsny, M., Kasah, R., Dehling, J.M., Proksch, S., Hödl, W., 2012. Multimodal communication in a noisy environment: a case study of the bornean rock frog,Staurois parvus. PLoS One 7, e37965.

Gray, G., Sassoon, D., Kelley, D., 1985. Sexual dimorphism of muscle fiber types in the larynx of Xenopus laevis. Soc. Neurosci. Abstr. 212.

Guerriero, G., 2009. Vertebrate sex steroid receptors: evolution, ligands, and neurodistribution. Ann. N. Y. Acad. Sci. 1163, 154–168.

Gurney, M.E., 1981. Hormonal control of cell form and number in the zebra finch song system. J. Neurosci. 1, 658–673.

Hau, M., 2007. Regulation of male traits by testosterone: implications for the evolution of vertebrate life histories. Bioessays 29, 133–144.

Hayes, T.B., 1998. Sex determination and primary sex differentiation in amphibians: genetic and developmental mechanisms. J. Exp. Zool. 281, 373–399.

Hiipakka, R.A., Liao, S., 1998. Molecular mechanism of androgen action. Trends Endocrino.Metab. 9, 317–324.

Hödl, W., Amézquita, A., 2001. Visual signaling in anuran amphibians. In: Ryan, M.J. (Ed.), Anuran Communication. Smithsonian.

Hoke, K.L., Adkins-Regan, E., Bass, A.H., McCune, A.R., Wolfner, M.F., 2019. Co-opting evo-devo concepts for new insights into mechanisms of behavioural diversity. J. Exp. Biol. 222.

Jin, J., Pawson, T., 2012. Modular evolution of phosphorylation-based signalling systems. Philos. Trans. R. Soc., B 367, 2540–2555.

Johnson, M.A., Kircher, B.K., Castro, D.J., 2018. The evolution of androgen receptor expression and behavior in Anolis lizard forelimb muscles. J. Comp. Physiol. A Neuroethol. Sens. Neural Behav. Physiol. 204, 71–79.

Jourjine, N., Hoekstra, H.E., 2021. Expanding evolutionary neuroscience: insights from comparing variation in behavior. Neuron 109, 1084–1099.

Kang, L., Marin, M., Kelly, D., 1995. Androgen biosynthesis and secretion in developing Xenopus laevis. Gen. Comp. Endocrinol. 100, 293–307.

Kaspar, B.K., Llado, J., Sherkat, N., Rothstein, J.D., Gage, F.H., 2003. Retrograde viral delivery of IGF-1 prolongs survival in a mouse ALS model. Science 301, 839–842.

Katz, P.S., Hale, M.E., 2017. Evolution of motor systems. In: Neurobiology of Motor Control: Fundamental Concepts and New Directions, pp. 135–176.

Kelley, D., Sassoon, D., Segil, N., Scudder, M., 1989. Development and hormone regulation of androgen receptor levels in the sexually dimorphic larynx of Xenopus laevis. Dev. Biol. 131, 111–118.

Kelley, D.B., Ballagh, I.H., Barkan, C.L., Bendesky, A., Elliott, T.M., Evans, B.J., Hall, I.C., Kwon, Y.M., Kwong-Brown, U., Leininger, E.C., 2020. Generation, coordination, and evolution of neural circuits for vocal communication. J. Neurosci. 40, 22–36.

Ketterson, E.D., Atwell, J.W., McGlothlin, J.W., 2009. Phenotypic integration and independence: hormones, performance, and response to environmental change. Integr. Comp. Biol. 49, 365–379.

Kirschner, M.W., Gerhart, J.C., 2008. The plausibility of life. In: The Plausibility of Life. Yale University Press.

Kobayashi, T., Nagahama, Y., 2009. Molecular aspects of gonadal differentiation in a teleost fish, the Nile tilapia. Sex.Dev. 3, 108–117.

Koopman, P., Gubbay, J., Vivian, N., Goodfellow, P., Lovell-Badge, R., 1991. Male development of chromosomally female mice transgenic for Sry. Nature 351, 117–121.

Kurz, E.M., Sengelaub, D.R., Arnold, A.P., 1986. Androgens regulate the dendritic length of mammalian motoneurons in adulthood. Science 232, 395–398.

Kusmierski, R., Borgia, G., Uy, A., Crozier, R.H., 1997. Labile evoltion of display traits in bowerbirds indicates reduced effects of phylogenetic constraint. Proc. R. Soc. Lond. B Biol. Sci. 264, 307–313.

Ladle, D.R., Pecho-Vrieseling, E., Arber, S., 2007. Assembly of motor circuits in the spinal cord: driven to function by genetic and experience-dependent mechanisms. Neuron 56, 270–283.

Ledon-Rettig, C.C., Pfennig, D.W., Crespi, E.J., 2010. Diet and hormonal manipulation reveal cryptic genetic variation: implications for the evolution of novel feeding strategies. Proc. R. Soc. B Biol. Sci. 277, 3569–3578.

Ledón-Rettig, C.C., Ragsdale, E.J., 2021. Physiological mechanisms and the evolution of plasticity. In: Phenotypic Plasticity & Evolution. CRC Press, pp. 113–137.

Ledón-Rettig, C.C., Pfennig, D.W., 2011. Emerging model systems in eco-evo-devo: the environmentally responsive spadefoot toad. Evol.Dev. 13, 391–400.

Leininger, E.C., Kelley, D.B., 2013. Distinct neural and neuromuscular strategies underlie independent evolution of simplified advertisement calls. Proc. R. Soc. Lond. B Biol. Sci. 280, 20122639.

Leininger, E.C., Kelley, D.B., 2015. Evolution of courtship songs in Xenopus: vocal pattern generation and sound production. Cytogenet.Genome Res. 145, 302–314.

Leininger, E.C., Kitayama, K., Kelley, D.B., 2015. Species-specific loss of sexual dimorphism in vocal effectors accompanies vocal simplification in African clawed frogs (Xenopus). J. Exp. Biol. 218, 849–857.

Lerner, D.T., Mason, R.T., 2001. The influence of sex steroids on the sexual size dimorphism in the red-spotted garter snake, Thamnophis sirtalis concinnus. Gen. Comp. Endocrinol. 124, 218–225.

Li, X.-M., Dong, X.-P., Luo, S.-W., Zhang, B., Lee, D.-H., Ting, A.K., Neiswender, H., Kim, C.-H., Carpenter-Hyland, E., Gao, T.-M., 2008. Retrograde regulation of motoneuron differentiation by muscle β-catenin. Nat. Neurosci. 11, 262–268.

Lipshutz, S., George, E., Bentz, A., Rosvall, K., 2019. Evaluating testosterone as a phenotypic integrator: from tissues to individuals to species. Mol. Cell. Endocrinol. 496, 110531.

London, S.E., Monks, D.A., Wade, J., Schlinger, B.A., 2006. Widespread capacity for steroid synthesis in the avian brain and song system. Endocrinology 147, 5975–5987.

London, S.E., Schlinger, B.A., 2007. Steroidogenic enzymes along the ventricular proliferative zone in the developing songbird brain. J. Comp. Neurol. 502, 507–521.

MacDougall-Shackleton, S.A., Ball, G.F., 1999. Comparative studies of sex differences in the song-control system of songbirds. Trends Neurosci. 22, 432–436.

Madison, F.N., Rouse Jr., M.L., Balthazart, J., Ball, G.F., 2015. Reversing song behavior phenotype: testosterone driven induction of singing and measures of song quality in adult male and female canaries (Serinus canaria). Gen. Comp. Endocrinol. 215, 61–75.

Mangiamele, L.A., Fuxjager, M.J., Schuppe, E.R., Taylor, R., Hodl, W., Preininger, D., 2016. Increased androgenic sensitivity in the hind limb neuromuscular system marks the evolution of a derived gestural display. Proc. Natl Acad. Sci. USA 113, 5664–5669.

Manolakou, P., Lavranos, G., Angelopoulou, R., 2006. Molecular patterns of sex determination in the animal kingdom: a comparative study of the biology of reproduction. Reprod. Biol. Endocrinol. 4, 1–23.

Massie, C.E., Adryan, B., Barbosa-Morais, N.L., Lynch, A.G., Tran, M.G., Neal, D.E., Mills, I.G., 2007. New androgen receptor genomic targets show an interaction with the ETS1 transcription factor. EMBO Rep. 8, 871–878.

McCarthy, M.M., 2016. Multifaceted origins of sex differences in the brain. Philos. Trans. R. Soc., B 371, 20150106.

McPhie-Lalmansingh, A.A., Tejada, L.D., Weaver, J.L., Rissman, E.F., 2008. Sex chromosome complement affects social interactions in mice. Horm. Behav. 54, 565–570.

Miles, M.C., Fuxjager, M.J., 2018. Synergistic selection regimens drive the evolution of display complexity in birds of paradise. J. Anim. Ecol. 87, 1149–1159.

Nakamura, M., 2010. The mechanism of sex determination in vertebrates—are sex steroids the key-factor? J. Exp. Zool. A Ecol. Genet. Physiol. 313, 381–398.

Nelson, R.J., Kreiegsfeld, L., 2015. An Introduction to Behavioiral Endocrinology. Sinauer, Sunderland.

Newman, A.E., Pradhan, D.S., Soma, K.K., 2008. Dehydroepiandrosterone and corticosterone are regulated by season and acute stress in a wild songbird: jugular versus brachial plasma. Endocrinology 149, 2537–2545.

Nottebohm, F., 1980. Testosterone triggers growth of brain vocal control nuclei in adult female canaries. Brain Res. 189, 429–436.

Nottebohm, F., 1981. A brain for all seasons: cyclical anatomical changes in song control nuclei of the canary brain. Science 214, 1368–1370.

Nottebohm, F., Arnold, A.P., 1976. Sexual dimorphism in vocal control areas of the songbird brain. Science 194, 211–213.

Ord, T.J., Blumstein, D.T., Evans, C.S., 2001. Intrasexual selection predicts the evolution of signal complexity in lizards. Proc. R. Soc. Lond. B Biol. Sci. 268, 737–744.

Ord, T.J., Blumstein, D.T., Evans, C.S., 2002. Ecology and signal evolution in lizards. Biol. J. Linn. Soc. 77, 127–148.

Oyegbile, T.O., Marler, C.A., 2005. Winning fights elevates testosterone levels in California mice and enhances future ability to win fights. Horm. Behav. 48, 259–267.

Oyegbile, T.O., Marler, C.A., 2006. Weak winner effect in a less aggressive mammal: correlations with corticosterone but not testosterone. Physiol. Behav. 89, 171–179.

Pease, J.B., Driver, R.J., de la Cerda, D.A., Day, L.B., Lindsay, W.R., Schlinger, B.A., Schuppe, E.R., Balakrishnan, C.N., Fuxjager, M.J., 2022. Layered evolution of gene expression in “superfast” muscles for courtship. Proc. Natl Acad. Sci. USA 119, e2119671119.

Phoenix, C.H., Goy, R.W., Gerall, A.A., Young, W.C., 1959. Organizing action of prenatally administered testosterone propionate on the tisues mediating mating behavior in the female guinea pig. Endocrinology 65, 369–382.

Pires-daSilva, A., Sommer, R.J., 2003. The evolution of signalling pathways in animal development. Nat. Rev. Genet. 4, 39–49.

Pollack, E.D., 1988. Perspectives on the development of the lateral motor column. In: Developmental Neurobiology of the Frog. Liss, New York, pp. 5–27.

Pradhan, D.S., Newman, A.E.M., Wacker, D.W., Wingfield, J.C., Schlinger, B.A., Soma, K. K., 2010. Aggressive interactions rapidly increase androgen synthesis in the brain during the non-breeding season. Horm. Behav. 57, 381–389.

Preininger, D., Boeckle, M., Freudmann, A., Starnberger, I., Sztatecsny, M., Hödl, W., 2013a. Multimodal signaling in the small torrent frog (Micrixalus saxicola) in a complex acoustic environment. Behav. Ecol. Sociobiol. 67, 1449–1456.

Preininger, D., Boeckle, M., Hödl, W., 2009. Communication in Noisy Environments II: Visual Signaling Behavior of Male Foot-flagging Frogs Staurois latopalmatus.

Preininger, D., Boeckle, M., Sztatecsny, M., Hödl, W., 2013b. Divergent receiver responses to components of multimodal signals in two foot-flagging frog species. PLoS One 8, e55367.

Preininger, D., Stiegler, M.J., Gururaja, K., Vijayakumar, S., Torsekar, V.R., Sztatecsny, M., Hödl, W., 2013c. Getting a kick out of it: multimodal signalling during male–male encounters in the foot-flagging frog Micrixalus aff. saxicola from the Western Ghats of India. Curr. Sci. 105, 1735–1740.

Preininger, D., Weissenbacher, A., Wampula, T., Hödl, W., 2012. The conservation breeding of two foot-flagging frog species from Borneo, Staurois parvus and Staurois guttatus. Amphibian Reptile Conserv. 5, 45–56.

Pryce, C.R., 2008. Postnatal ontogeny of expression of the corticosteroid receptor genes in mammalian brains: inter-species and intra-species differences. Brain Res. Rev. 57, 596–605.

Quartier, A., Chatrousse, L., Redin, C., Keime, C., Haumesser, N., Maglott-Roth, A., Brino, L., Le Gras, S., Benchoua, A., Mandel, J.-L., 2018. Genes and pathways regulated by androgens in human neural cells, potential candidates for the male excess in autism spectrum disorder. Biol. Psychiatry 84, 239–252.

Rand, M.N., Breedlove, S.M., 1995. Androgen alters the dentritic arbors of SNB motoneurons by acting upon their target muscles. J. Neurosci. 15, 4408–4416.

Regnier, M., Herrera, A.A., 1993. Changes in the contractile properties by androgen hormones in sexually dimorphic muscles of male frogs (Xenopus laevis). J.Physiol. 461, 565–581.

Rosvall, K.A., Bergeon Burns, C., Jayaratna, S., Dossey, E., ED, K., 2016. Gonads and the evolution of hormonal phenotypes. Integr. Comp. Biol. 56, 225–234.

Rosvall, K.A., Burns, C.M.B., Barske, J., Goodson, J.L., Schlinger, B.A., Sengelaub, D.R., Ketterson, E.D., 2012. Neural sensitivity to sex steroids predicts individual differences in aggression: implications for behavioural evolution. Proc. R. Soc. Lond. B Biol. Sci. 279, 3547–3555.

Ryan, M.J., 2021. Evolution of behavior. In: The Behavior of Animals, 2nd editionMechanisms, Function and Evolution, pp. 397–426.

Saltiel, P., Wyler-Duda, K., D'Avella, A., Tresch, M.C., Bizzi, E., 2001. Muscle synergies encoded within the spinal cord: evidence from focal intraspinal NMDA iontophoresis in the frog. J. Neurophysiol. 85, 605–619.

Sanger, T.J., Rajakumar, R., 2019. How a growing organismal perspective is adding new depth to integrative studies of morphological evolution. Biol. Rev. 94, 184–198.

Sarre, S.D., Georges, A., Quinn, A., 2004. The ends of a continuum: genetic and temperature-dependent sex determination in reptiles. Bioessays 26, 639–645.

Sassoon, D.A., Gray, G.E., Kelley, D.B., 1987. Androgen regulation of muscle fiber type in the sexually dimorphic larynx of Xenopus laevis. J. Neurosci. 7, 3198–3206.

Sassoon, D.A., Segil, N., Kelley, D.B., 1986. Androgen induced myogenesis and chondrogenesis in the larynx of Xenopus laevis. Dev. Biol. 113, 135–140.

Schlinger, B.A., Brenowitz, E.A., 2002. Neural and hormonal control of birdsong. Hormones Brain Behav. 799–839.

Schlosser, G., 2003. Mosaic evolution of neural development in anurans: acceleration of spinal cord development in the direct developing frog Eleutherodactylus coqui. Anat. Embryol. 206, 215–227.

Schuppe, E.R., Miles, M.C., Fuxjager, M.J., 2020. Evolution of the androgen receptor: perspectives from human health to dancing birds. Mol. Cell. Endocrinol. 499, 110577.

Schuppe, E.R., Tobiansky, D.J., Goller, F., Fuxjager, M.J., 2022. Specialized androgen synthesis in skeletal muscles that actuate elaborate social displays. J. Exp. Biol. 225, jeb243730.

Schwark, R.W., Fuxjager, M.J., Schmidt, M.F., 2022. Proposing a neural framework for the evolution of elaborate courtship displays. eLife 11, e74860.

Segil, N., Silverman, L., Kelley, D.B., 1987. Androgen-binding levels in a sexually dimorphic muscle of Xenopus laevis. Gen. Comp. Endocrinol. 66, 95–101.

Sekido, R., Lovell-Badge, R., 2009. Sex determination and SRY: down to a wink and a nudge? Trends Genet. 25, 19–29.

Sheng, Z., Kawano, J., Yanai, A., Fujinaga, R., Tanaka, M., Watanabe, Y., Shinoda, K., 2004. Expression of estrogen receptors (α, β) and androgen receptor in serotonin neurons of the rat and mouse dorsal raphe nuclei; sex and species differences. Neurosci. Res. 49, 185–196.

Smith, S.M., Eigerman, A.R., LeCure, K.M., Kironde, E., Privett-Mendoza, A.G., Fuxjager, M.J., Preininger, D., Mangiamele, L.A., 2021. Androgen receptor modulates multimodal displays in the Bornean rock frog (Staurois parvus). Integr. Comp. Biol. 61, 221–230.

Soma, K., Scotti, M.L., Newman, A.E.M., Charlier, T.D., Demas, G.E., 2008. Novel mechanisms for neuroendocrine regulation of aggression. Front. Neuroendocrinol. 29, 476–489.

Song, J., Ampatzis, K., Bjornfors, E.R., El Manira, A., 2016. Motor neurons control locomotor circuit function retrogradely via gap junctions. Nature 529, 399–402.

Sperry, D.G., Grobstein, P., 1985. Regulation of neuron numbers in Xenopus laevis: effects of hormonal manipulation altering size at metamorphosis. J. Comp. Neurol. 232, 287–298.

Stehouwer, D.J., Farel, P.B., 1984. Development of hindlimb locomotor behavior in the frog. Dev. Psychobiol. 17, 217–232.

Stehouwer, D.J., Farel, P.B., 1985. Development of locomotor mechanisms in the frog. J. Neurophysiol. 53, 1453–1466.

Tobiansky, D.J., Fuxjager, M.J., 2020. Sex steroids as regulators of gestural communication. Endocrinology 161, bqaa064.

Tobiansky, D.J., Miles, M.C., Goller, F., Fuxjager, M.J., 2020. Androgenic modulation of extraordinary muscle speed creates a performance trade-off with endurance. J. Exp. Biol. 223.

Tobias, M.L., Kelley, D.B., 1987. Vocalizations by a sexually dimorphic isolated larynx: peripheral constraints on behavioral expression. J. Neurosci. 7, 3191–3197.

Tobias, M.L., Marin, M.L., Kelley, D.B., 1991. Development of functional sex differences in the larynx of Xenopus laevis. Dev. Biol. 147, 251–259.

Uy, J.A.C., Borgia, G., 2000. Sexual selection drives rapid divergence in bowerbird display traits. Evolution 54, 273–278.

Verhovshek, T., Sengelaub, D.R., 2013. Androgen action at the target musculature regulates brain-derived neurotrophic factor protein in the spinal nucleus of the bulbocavernosus. Dev.Neurobiol. 73, 587–598.

Watson, J., Robertson, J., Sachdev, U., Kelley, D., 1993. Laryngeal muscle and motor neuron plasticity in Xenopus laevis: analysis of a sensitive period for testicular masculinization of a neuromuscular system. J. Neurobiol. 24, 1615–1625.

Watson, J.T., Kelley, D.B., 1992. Testicular masculinization of vocal behavior in juvenile female Xenopus laevis reveals sensitive periods for song duration, rate, and frequency spectra. J. Comp. Physiol. A. 171, 343–350.

Westneat, D., Fox, C.W., Fox, C., 2010. Evolutionary Behavioral Ecology. Oxford University Press.

Williams, T.D., 2008. Individual variation in endocrine systems: moving beyond the 'tyranny of the Golden Mean'. Philos. Trans. R. Soc. Lond. B Biol. Sci. 363, 1687–1698.

Wittman, T.N., Robinson, C.D., McGlothlin, J.W., Cox, R.M., 2021. Hormonal pleiotropy structures genetic covariance. Evol.Lett. 5, 397–407.

Wolff, J.O., Cicirello, D.M., 1990. Mobility versus territoriality: alternative reproductive strategies in white-footed mice. Anim. Behav. 39, 1222–1224.

Wu, K.H., Tobias, M.L., Kelley, D.B., 2001. Estrogen and laryngeal synaptic strength in Xenopus laevis: opposite effects of acute and chronic exposure. Neuroendocrinology 74, 22–32.

Young, L.J., Crews, D., 1995. Comparative neuroendocrinology of steroid receptor gene expression and regulation: relationship to physiology and behavior. Trends Endocrinol.Metab. 6, 317–323.