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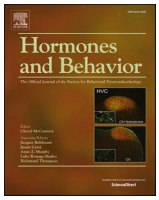

How new communication behaviors evolve: Androgens as modifiers of neuromotor structure and function in foot-flagging frogs

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ABSTRACT

How diverse animal communication signals have arisen is a question that has fascinated many. *Xenopus* frogs have been a model system used for three decades to reveal insights into the neuroendocrine mechanisms and evolution of vocal diversity. Due to the ease of studying central nervous system control of the laryngeal muscles in vitro, *Xenopus* has helped us understand how variation in vocal communication signals between sexes and between species is produced at the molecular, cellular, and systems levels. Yet, it is becoming easier to make similar advances in non-model organisms. In this paper, we summarize our research on a group of frog species that have evolved a novel hind limb signal known as 'foot flagging.' We have previously shown that foot flagging is androgen dependent and that the evolution of foot flagging in multiple unrelated species is accompanied by the evolution of higher androgen hormone sensitivity in the leg muscles. Here, we present new preliminary data that compare patterns of androgen receptor expression and neuronal cell density in the lumbar spinal cord – the neuromotor system that controls the hind limb – between foot-flagging and non-foot-flagging frog species. We then relate our work to prior findings in *Xenopus*, highlighting which patterns of hormone sensitivity and neuroanatomical structure are shared between the neuromotor systems underlying *Xenopus* vocalizations and foot-flagging frogs' limb movement and which appear to be species-specific. Overall, we aim to illustrate the power of drawing inspiration from experiments in model organisms, in which the mechanistic details have been worked out, and then applying these ideas to a non-model species to reveal new details, further complexities, and fresh hypotheses.

1. Introduction

Diversity in sexual communication behaviors has fascinated biologists since the time of Darwin. Whether it is the colorful and elaborate mating displays of birds of paradise or the complex vocalizations of male túngara frogs calling for a mate, the question of *how* evolution has produced these "endless forms most beautiful" (Darwin 1859, 490) has been one that scientists in several fields have aimed to tackle. In recent decades, behavioral neuroendocrinologists have turned their attention to this problem by investigating the role that sex steroid hormones, such as androgens and estrogens, play in the evolution of diverse sexual phenotypes (Anderson et al., 2022; Cox, 2020a; Fusani et al., 2014; Fuxjager et al., 2015; Fuxjager and Schlinger, 2015; Fuxjager and Schuppe, 2018; Hau, 2007; Ketterson et al., 2009; Lipshutz et al., 2019; Mangiamele and Fuxjager, 2018; Wingfield et al., 1990, 2020). From these studies, two key ideas have emerged that have informed our understanding of the mechanisms associated with diversity in communication signals.

First, sex differences in communication signals can arise via the evolution of sexual dimorphisms in hormonal factors, such as circulating hormone levels, enzymes that locally metabolize sex steroids, or hormone receptor distribution in tissues (Fuxjager et al., 2018; Fuxjager and Schuppe, 2018; Ho et al., 2013). In many vertebrate species, testosterone (T) is the predominant gonadal hormone secreted in males

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(although in teleost fish 11-ketotestosterone is equally important). One way that T can promote sex differences in signaling behavior is by directing the development of male signaling structures, and their underlying neuromotor circuits, and by maintaining sexual dimorphisms in these structures throughout the lifespan. In adulthood, T is also one of the major factors that influences how males express their communication signals (Adkins-Regan, 2005; Alward et al., 2018; Ball, 2003; Day et al., 2007; Dunlap et al., 1998; Fusani, 2008; Remage-Healey and Bass, 2006; Wade, 2012; Zornik and Kelley, 2011). For example, in some seasonally breeding songbirds, elevated T levels in males correspond to increased time spent singing, song duration, complexity, sterotypy, and vocal performance (e.g., trill rate) (reviewed in Ball et al., 2017; however, there are notable exceptions, particularly in songbird species in which females also sing. See Ball, 2016; Riebel et al., 2019; Schwabl et al., 2015). Historically, these phenomena have been primarily investigated in species in which the production of sexual communication signals is highly sexually dimorphic and male-biased. However, as more species are studied, we see that variation in behavioral sex differences – or the degree and nature of sexual dimorphism in a given species compared with another species – is also associated with variation in the action of androgens on signal-producing structures (Ball, 2016; Dunlap et al., 1998; Lipshutz and Rosvall, 2020; Smith, 2013). Thus, the evolution of sexual diversity in communication signals is often related to the evolution of differences in steroid-driven effects.

Second, when communication repertoires diversify between species, there is often evidence of selection on androgen-mediated traits that are involved in the production of signals (e.g., Frankl-Vilches and Gahr, 2018; Shaw and Kennedy, 2002; Wilczynski et al., 1993), although this issue has been far less studied than differences between the sexes. In particular, recent work using phylogenetic approaches provides evidence that suggests that the level of androgen sensitivity in signal-associated tissues is positively related to the degree of complexity of males' display. For example, bird species with more complex physical displays have higher levels of androgen receptor (AR) in their wing musculature (Fuxjager et al., 2015; Fuxjager and Schlinger, 2015), and lizard species that perform more push up displays have more AR in their fore limb muscles (Johnson et al., 2018). These studies suggest that the same mechanisms that have evolved to generate sex differences in signaling behavior may also be at play when species differences in social communication emerge.

Studies in anuran amphibians (frogs and toads) have been particularly important in advancing our understanding of the mechanisms and evolution of communication. In particular, work in *Xenopus laevis* (South African clawed frog), which was initiated three decades ago by Darcy Kelley, has provided a vertebrate model in which to study vocal communication in the laboratory at the molecular, cellular, systems, and organismal levels (reviewed in Kelley et al., 2020). Male *Xenopus laevis* produce trill-like courtship vocalizations by vibrating the muscles of the larynx, but females do not make these vocalizations (Kelley and Tobias, 1999). By integrating observational studies of frog behavior with experiments using isolated preparations of brain and larynx, *Xenopus* researchers have described how specific neuromotor circuits generate sex- and species-specific vocal patterns, and how these patterns are influenced by hormones and other neuromodulators. The availability of a well-supported phylogeny, as well as several reference genomes, has also made the *Xenopus* family a model for studies of vocal evolution between species. For example, comparative work has used examples of parallel and divergent evolution of vocal characters to identify specific changes in the physiology of the vocal motor system that underlie changes in calling behavior (Barkan et al., 2017, 2018; Leininger et al., 2015; Leininger and Kelley, 2013). Together, these studies have been instrumental in providing a mechanistic understanding of the neuroendocrine factors underlying vocal behavior, along with insights into evolutionary processes.

Yet, are these insights that can apply more broadly to other cases of signal evolution, or are they features of a nervous system specialized for vocalization? In the field of animal communication, work on the evolution of signal production mechanisms has been focused largely on acoustic signaling (e.g., see the extensive literature on birdsong and frog vocalizations), perhaps because these behaviors can be most easily elicited and manipulated in the laboratory. The mechanisms and evolution of other signaling behaviors have not been as well studied (but see Freiler and Smith, 2023; Khalil et al., 2023; Proffitt et al., 2023; Smith, 2013). This gap leaves open questions about the different paths that evolution can take in shaping communication behavior. Are there common mechanisms by which neuromotor pathways are modified by evolution for signal production? Are there evolutionary constraints that are shared – for example, conserved neurodevelopmental mechanisms? Or are there multiple ways through which a new communication signal can evolve?

To answer these questions, we need to study more species with more diverse communication repertoires. In particular, model systems that can help us test hypotheses about the causes of variation between species are needed to help us move beyond single case studies and toward the discovery of a set of general principles by which communication signals may evolve (Gallant and O'Connell, 2020; Jourjine and Hoekstra, 2021). To begin to address this need, we have studied a group of non-model organisms, foot-flagging frogs, which has allowed us to extend and expand upon what we've learned about the neuroendocrine mechanisms of signal evolution in *Xenopus* (Anderson et al., 2022; Mangiamele and Fuxjager, 2018). Foot flagging is a gestural signal that has evolved in roughly two dozen frog species from across the anuran phylogeny (Fig. 1). Males perform a foot flag by extending and conspicuously waving their hind limb, while displaying brightly colored foot webbing, in order to deter sexual rivals and possibly attract mates (Grafe et al., 2012; Hödl and Amezquita, 2001; Preininger et al., 2009, 2013a, 2013b, 2013c). Although there is some diversity among foot-flagging frog species, their ecology and behavior are dramatically different from *Xenopus* frogs. In contrast to *Xenopus*, which are fully aquatic, all foot-flagging frogs live terrestrial lifestyles. Most species live in noisy environments beside rushing streams and waterfalls that make acoustic communication difficult. Thus, the sensory landscape for communication exerts very different evolutionary pressures on *Xenopus* and foot-flagging frogs. In addition, foot flagging is just one of the most prominent signals in the frogs' multimodal display repertoire. Other signaling behaviors that have been observed include opening the mouth to reveal bright coloration, upright posturing to display a white underbelly, inflating the vocal sac, and the production of high-pitched vocalizations (Grafe et al., 2012; Preininger et al., 2009).

The evolution of foot flagging is clear: in all cases it is a derived character that emerges after acoustic communication. Foot flagging in frogs is also a classic example of convergence, having arisen independently at least 5 times in different anuran families, presumably to overcome the challenge of acoustic signaling in especially noisy environments. (Anderson et al., 2023; Hödl and Amezquita, 2001; Fig. 1). These phylogenetic relationships have enabled comparative studies, which show that the emergence of the foot flag in multiple frog species is marked by a similar dramatic increase in androgen receptor expression in the hind limb muscles (Anderson et al., 2021b; Mangiamele et al., 2016). This finding suggests that foot-flagging frogs' communication repertoire is expanded by selection acting on the output of the hind limb neuromotor system. In fact, one advantage of this non-model organism is that foot flagging is generated by a neuromotor system that, unlike the *Xenopus* vocal motor system, is not specialized for sexual signaling, but rather is multifunctional and also used for locomotion. This provides an opportunity to reveal the mechanisms by which evolution has co-opted the hind limb neuromotor system for producing sexual signals (Mangiamele and Fuxjager, 2018). Thus, foot-flagging frogs are an excellent model in which to focus on androgen's role in supporting the emergence of a completely novel communication signal and to begin working to identify its tissue-specific actions on the neuromotor system that controls it.

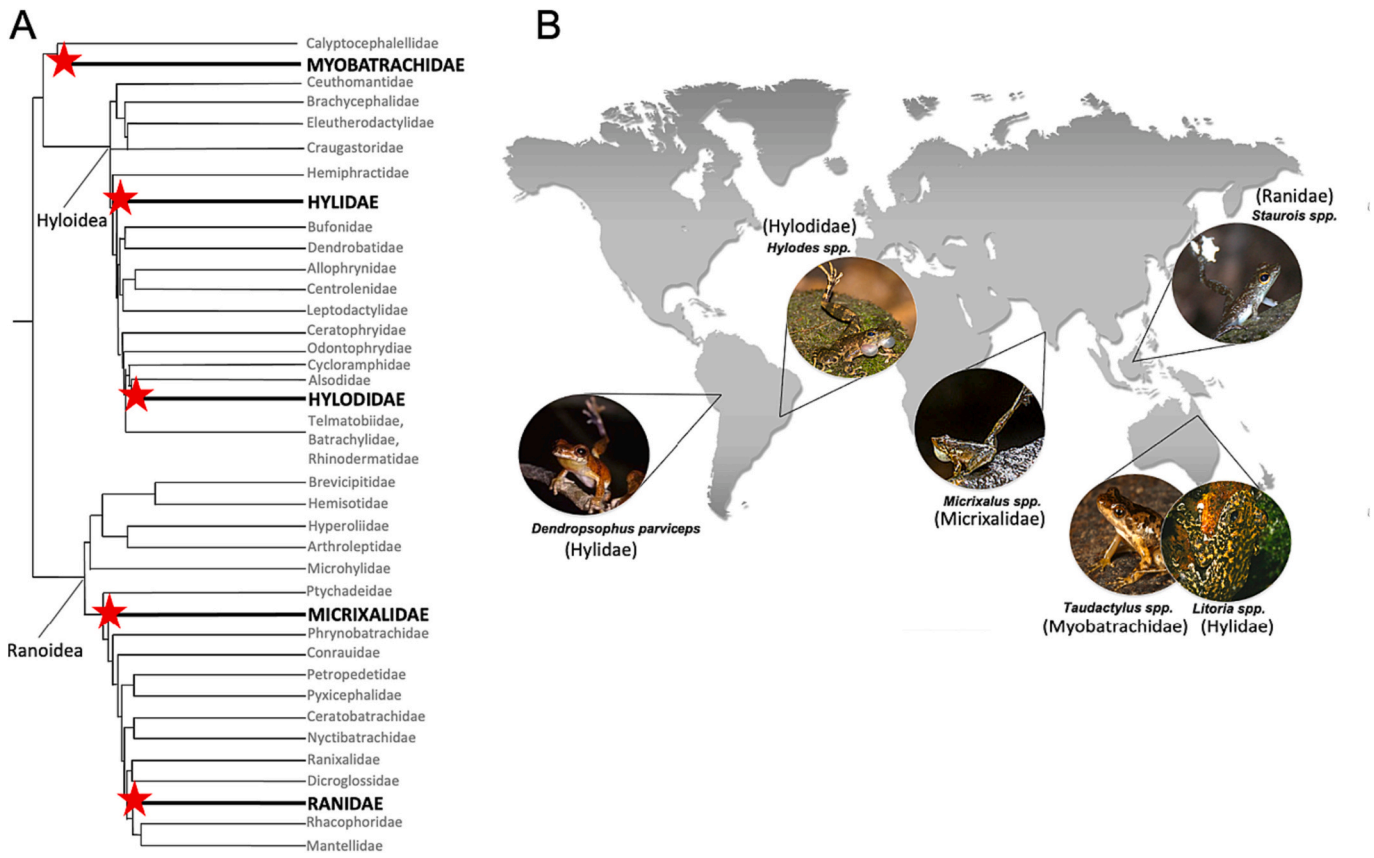

Fig. 1. Foot flagging has arisen independently multiple times in the anuran phylogeny. (A) Family-level phylogeny with independent evolution of foot-flagging behavior denoted with stars. Other branches of the tree represent non-foot-flagging anuran families in which vocalizations are the primary sexual signal (grey text). Adapted from Holtz et al., 2023. (B) Map showing geographic distribution of diverse foot-flagging frog species. Family names are in parentheses. (Photo credits: *Staurois parvus*, Daniel Zupanc; all others Creative Commons).

In this article, we first briefly review what is known about the mechanisms of *Xenopus* vocal evolution, which leads us to questions that remain open for further investigation. We then summarize our own work on foot-flagging frogs, highlighting patterns that emerge when we compare frogs with the novel hind limb signal to those species that have not evolved the signal. Our previous work has focused on species comparisons of androgenic sensitivity in the hind limb musculature. Here, we present previously unpublished data that may reveal how the spinal circuitry that controls the hind limb, but not the fore limb, could differ in foot-flagging frogs compared to species that do not foot flag. Finally, we draw comparisons between the insights we have gained in *Xenopus* and foot-flagging frogs. We point out where research findings have converged and where there are differences. Overall, we aim to illustrate the power of drawing inspiration from experiments in model organisms, in which the mechanistic details of steroid action on neural systems for communication have been worked out, and then applying these ideas to a non-model species to reveal new details, further complexities, and fresh hypotheses. These advances have provided fertile ground for further comparative work, with the ultimate goal of uncovering general evolutionary principles that can be applied to a greater range of species.

2. Neuroendocrine mechanisms of vocal diversity: insights from *Xenopus*

Adult *Xenopus* have a diverse vocal repertoire. These vocalizations vary primarily in the temporal patterning of "clicks" of sound that are generated by the contraction and relaxation of paired laryngeal muscles. Males can make five different types of vocalizations, of which the rapidly trilled (i.e., a series of clicks of up to 70 Hz) courtship call is the best studied (Tobias et al., 2004). One type of vocalization, a slow (6 Hz) series of clicks known as "ticking", can be produced by both sexes (Tobias et al., 1998). Male clicks are also higher pitch compared to female clicks (Tobias et al., 1998, 2004).

Research in *Xenopus* has helped illuminate some of the basic mechanisms by which hormones may drive this behavioral diversity. That work has been reviewed in detail elsewhere (Kelley et al., 2020; Zornik and Kelley, 2011; Zornik and Yamaguchi, 2008), so here we will only briefly highlight research on androgen-dependent vocal behavior that has inspired our own. Sex differences in *Xenopus* communication signaling arise after a developmental stage in which the level of circulating testosterone begins to increase dramatically in males, but not females (Kang et al., 1995). Mature males gain the ability to produce courtship calls but retain the ability to produce ticks. Notably, *Xenopus* were among the first organisms in which it was demonstrated that androgens are necessary for male courtship signals (Wetzel and Kelley, 1983). Castration abolishes, but T treatment restores, normal courtship calling in adult males (Kelley and Pfaff, 1976; Wetzel and Kelley, 1983; Zornik and Yamaguchi, 2011). Long-term treatment with the androgen receptor antagonist, flutamide, also decreases calling behavior in males (Behrends et al., 2010).

2.1. Androgen regulation of pattern-generating neuronal circuitry

Androgens facilitate differences in the vocalizations of *Xenopus* males and females by directing the organization and sexual differentiation of neural circuits in the vocal motor system that are required for producing rapid trills. The brain regions and nerves that control the sound-producing laryngeal muscles express high levels of androgen receptors

in adult males, while females' brains are relatively insensitive to androgens (Kelley, 1980; Perez et al., 1996; Wetzel and Kelley, 1983). Numerous studies have documented androgen-driven differences in the morphological features of these neural circuits in males compared to females, such as increased complexity of dendritic branching patterns of motoneurons innervating the larynx (Kelley et al., 1988), increases in the number of motoneurons (Kay et al., 1999; Watson et al., 1993), and increases in motoneuron soma size (Potter et al., 2005; Yamaguchi et al., 2003). Male and female motoneurons also differ in their cellular physiological properties (Yamaguchi et al., 2003).

Importantly, androgens promote a gain of function in the neural circuitry underlying vocal behavior in *Xenopus* males. While adult females only have one circuit controlling vocalization, adult males appear to have two. Detailed in vitro studies of neural connectivity and cellular physiology show that androgens are primarily responsible for adding a functional circuit for trilled vocalizations to the male central nervous system, which is distinct from the one that generates the ticking pattern (reviewed by Zornik and Yamaguchi, 2008). The result is that both male and female juveniles share a similar ticking call, but only adult males go on to develop the ability to produce the fast-paced trills of the courtship call. Applied broadly, T's ability to reshape the anatomy, physiology, and function of neural circuits likely makes this hormone's action a target of selection for behavioral evolution and sexual differentiation.

Work in female *Xenopus* demonstrates, however, that the neuromotor system controlling vocal production exhibits a generous amount of plasticity. Juvenile females treated with T can develop larger and more numerous, male-like motoneurons (Kay et al., 1999; Potter et al., 2005), which produce trilled vocalizations. However, their calling behavior never completely matches that of males (Potter et al., 2005; Watson and Kelley, 1992), suggesting that there is some factor other than androgens that also influences the development of vocal behavior in males. Nevertheless, the fact that females, given the right endocrine context, can develop vocal features similar to that of adult males illustrates the positive relationship between signal elaboration and androgens.

2.2. Androgen regulation of sound-producing muscle

Androgens also play a large role in shaping the peripheral structures that generate vocalizations in *Xenopus*. Laryngeal muscles in adult males are more sensitive to androgenic hormones and are highly sexually dimorphic (Kelley et al., 1989). Male laryngeal mass (Sassoon and Kelley, 1986), fiber type (Sassoon et al., 1987), myosin heavy chain isoform (Baur et al., 2008), and contractile properties (Tobias and Kelley, 1987) are different from that of females and have evolved to support the production of the sustained, rapid succession of clicks in courtship calls. For example, the muscle fibers in adult male larynges are all fast-twitch, meaning that they can sustain prolonged and rapid male vocalizations. By contrast, female fibers are mostly slow twitch (Marin et al., 1990; Sassoon et al., 1987). Similar to its effects in the central nervous system, T administered to juvenile females will cause them to develop laryngeal muscles that are both morphologically and functionally similar to males' (Potter et al., 2005; Tobias and Kelley, 1987). The mechanism for this androgen-induced fiber type switching in *Xenopus* is likely via a unique androgen receptor isoform expressed in juvenile stem cells that facilitates cell proliferation, differentiation, and gene expression of a male-specific muscle myosin (Catz et al., 1992, 1995; Nasipak and Kelley, 2012).

2.3. Evolution of vocal diversity in the Xenopus clade

Comparative studies of multiple species in the *Xenopus* genus have revealed the evolution of the neuromuscular substrates underlying differences in vocalization between species, but the story is complicated. Some studies have found differences in neural spiking patterns and membrane ion currents that may underlie the differences in vocal patterning between species (*X. laevis and X. petersi:* Barkan et al., 2017, 2018), but whether these cellular-level changes are the only source of evolutionary divergence in the communication signals of these two species is still unclear. The best look at the mechanisms of parallel evolution in *Xenopus* vocalizations compared two species (*X. boumbaensis* and *X. borealis*) in which males independently evolved a simpler, single click type courtship call. That study found that ***different*** neural firing patterns, laryngeal muscle contraction patterns, and laryngeal muscle morphology (i.e., percent fast/slow twitch fibers) had evolved in each species (Leininger et al., 2015; Leininger and Kelley, 2013). Kelley et al. (2020) speculate that single click type vocalizations in *X. borealis* males might have evolved via loss of androgen-regulated growth of the fast twitch fiber type, but thus far this hypothesis has not been tested.

In addition, laryngeal muscle morphology and physiology differ between sexes and species, and these patterns are not always clear. In each *Xenopus* species studied to date, there is a sexual dimorphism in laryngeal muscle mass, with males having larger larynges, that corresponds to a sexual dimorphism in laryngeal physiology. But the degree to which male and female larynges differ ranges from a lot to a little, and two species (*X. muelleri* and *X. borealis*) show sex differences in muscle mass but comparatively little differences in contractile properties, which underlie call features such as inter-pulse interval (Leininger et al., 2015; South et al., 2021). One possible mechanism underlying this subtle diversity in sexual dimorphism among species could be variation in the degree of androgen sensitivity of the laryngeal tissue and/or differences in developmental factors that are androgen-sensitive. For instance, selective losses of certain hormonally-regulated physiological properties, but not others, could lead to subtle differences in physiology and vocal features, including the creation of intermediate phenotypes, such as *X. muelleri* (South et al., 2021). Further work is necessary to help clarify the endocrine basis, if any, of the complex evolutionary patterns that are observed across the *Xenopus* phylogeny.

Overall, the extensive body of research in *Xenopus* points to several ways that the central nervous system and peripheral muscles controlling vocal production can diversify to generate communication signals that differ between sexes and species. Importantly, we see that hormone-induced variations do largely correlate with variation in vocal output. Yet, substantial questions remain, such as:

1. In the *Xenopus* frog lineage, parallel evolution of vocal signals does not always correspond to parallelism in the underlying morphology and physiology, and vice versa. There seem to be multiple mechanisms to solve the same problem. Is this a unique feature of a neuromotor system specialized for vocalization, which may be relatively unconstrained by evolutionary forces other than sexual selection? Do we see a similar diversity of structure-function relationships in other neuromotor systems when their communication signals converge?
2. In *Xenopus*, androgens influence multiple levels of the vocal motor system to generate differences in vocal signals, from laryngeal muscles to central pattern-generating neurons. Is this a common mechanism by which the evolution of all types of communication signals proceeds? Or might selection favor different mechanisms in different neuromotor systems?

These questions are not necessarily novel or unique to study of the evolution of frog communication (Adkins-Regan, 2007; Cox, 2020b; Hau, 2007; Lipshutz et al., 2019; Rosvall, 2022). Using the *Xenopus* system as inspiration, our work aims to address such long-standing questions and to produce unique insights into what mechanisms underlie the evolution of a novel communication signal in foot-flagging frogs, a group of frogs that use gestural signaling as the primary means of communication.

3. Neuroendocrine mechanisms accompanying evolution of a novel communication signal: foot-flagging in frogs

The best studied foot-flagging frog is the Bornean rock frog (*Staurois parvus*). In *S. parvus*, foot-flagging behavior in adult males is androgen dependent. We have shown that T increases the frequency of foot flagging, while administration of flutamide blocks this effect (Mangiamele et al., 2016; Smith et al., 2021), suggesting a key role for androgen receptors in regulating the production of this novel signal. In contrast to *Xenopus* and other frogs, however, the production of vocalizations does not seem to be affected by T, at least when observed within a few hours of its administration (Mangiamele et al., 2016). This result is interesting from an evolutionary perspective, as it appears to be the derived rather than the ancestral signal that is more strongly androgen modulated in *S. parvus*. However, given that the social and/or abiotic environment undoubtedly plays a role in a male's decision to produce foot flags or vocalizations (Grafe and Tony, 2017), further experiments are necessary to confirm whether T's lack of effect on vocalizations is context-specific. Nevertheless, our work in *S. parvus* represents the first time that a hormone's effects on a gestural signal has been described in frogs.

Androgens also modify the way that males produce their foot flags. Individual foot flags can be broken down into five distinct movement phases of the hind limb: (i) leg lift, (ii) full leg extension, (iii) arching posterior hip rotation, (iv) pull in leg toward the body, and (v) lower the foot. Anderson et al. (2021a) found that the foot flags of T-treated adult males were more circular in shape, and T modified the speed of some of the component movements (faster rotation, slower pull-in). Androgens are also important for the integration of foot flags into a male's broader multimodal display. Using network analysis, we revealed shifts in multimodal display architecture when T-treated males were also given the anti-androgen flutamide. We found that androgenic action appears necessary for a greater diversity of signal transitions in males' multimodal displays; males not treated with flutamide did more gestural/visual signaling (foot flags, upright posturing) and incorporated more complex behavioral sequences in their display bouts compared to flutamide-treated males (Eigerman and Mangiamele, 2022). Together, these findings suggest that fine motor control of the foot flag and the precise execution of multimodal behavioral routines, via brain and/or spinal cord circuits, is regulated by androgenic hormones.

3.1. Tissue-specific androgen sensitivity marks the evolution of foot-flagging in frogs

A major contribution of work in foot-flagging frogs is the discovery that the evolution of foot flagging coincides with increased androgen sensitivity in the hind limb thigh muscles that produce the gestural signal. This pattern was demonstrated by comparing AR levels in *S. parvus* muscle tissue to that of well studied non-foot-flagging frogs, *Rana (Lithobates) pipiens*[2] and *Xenopus laevis*, using qPCR (Mangiamele et al., 2016). A similarly elevated level of AR expression has also been found in two unrelated frog genuses that have convergently evolved foot-flagging behavior (*Micrixalus, Dendropsophus*) compared to their non-foot-flagging frog relatives (Anderson et al., 2021b). Notably, in contrast to large differences in AR in the brains of male vs. female *Xenopus*, a number of studies have not found that androgen sensitivity differs substantially in the central nervous systems of foot-flagging frogs compared to non-foot-flagging species. For example, phylogenetic analyses have found no association between the emergence of foot flagging and differences in AR level in the brain and spinal cord (Anderson et al., 2021b). When separating out brain from spinal cord, Mangiamele et al. (2016) found that overall levels of AR in the spinal cord of *S. parvus* did not differ from *R. pipiens*, but were higher than *X. laevis*. Together, this evidence supports the hypothesis that selection for foot flagging in multiple frog taxa has driven similar changes in the androgenic system, primarily in the peripheral signal-generating muscles.

Further, our work has demonstrated that *S. parvus* has a unique endocrine phenotype when compared with frogs that do not produce foot flags. While male *R. pipiens*, *X. laevis*, and *S. parvus* all produce vocalizations and, accordingly, all maintain equally high levels of AR in their laryngeal muscles, we have shown that the relative proportion of AR expressed by an individual in its leg musculature and larynx corresponds to the type of displays each species uses in their signaling repertoire. Frogs who use primarily vocal communication show far more AR in their larynx than leg muscles, while *S. parvus* individuals show equal partitioning (Mangiamele et al., 2016). This tissue-specific AR expression emphasizes the idea that testosterone can differentially affect multiple neuromotor systems in the same individual to influence specific behaviors.

Given that we have not previously found that an increase in overall AR levels in the central nervous system is associated with the evolution of foot-flagging in frogs, we wondered whether there were changes in androgen sensitivity that were specific to the cells in the lumbar spinal cord that make up the hind limb circuitry in *S. parvus.* As mentioned previously, the evolution of more complex vocal patterns in male *Xenopus* is associated with a number of changes in the hindbrain pattern generating circuitry, including higher AR expression and an increased number of motoneurons compared to females. We therefore hypothesized that a similar mechanism might be associated with the evolution of foot flagging, such that (i) foot-flagging frogs would have higher AR expression in their lumbar spinal cord compared to frogs that do not use their hind limbs for sexual signaling, and (ii) within *S. parvus* individuals AR expression would be higher in spinal cord neurons that control the hind limb muscles (lumbar) compared to those that control the fore limb muscles (brachial). We also hypothesized that the lumbar spinal cord in foot-flagging frogs differs anatomically from that in non-foot-flagging frogs, perhaps having more motoneurons and/or interneurons with which to exert finer control over the precise movements of the hind limb.

3.2. Species comparisons of androgen receptor in the spinal cord

To test the first hypothesis, we conducted an AR in situ hybridization to compare the distribution of AR in the spinal cord in three frog species: *S. parvus* and two non-foot-flagging frogs (*R. pipiens, X. laevis*). We compared AR expression among species in the brachial spinal cord, which controls fore limb movements, to that in the lumbar spinal cord, which controls hind limb movements.

3.2.1. Methods

To do this, reproductively active adult male *S. parvus* were sampled from a semi-wild population at the Vienna Zoo, Vienna, Austria, while reproductively active adult male *R. pipiens* and *X. laevis* were obtained from commercial suppliers (eNasco and Xenopus Express, respectively). All frogs were euthanized within two weeks of arriving at our laboratory. We collected whole spinal cord and brain tissue from 3 to 5 individuals per species. To quantify AR mRNA expression in the spinal cord, we first identified androgen receptor mRNA sequence in all three species and generated three separate, species-specific radioactively (^{35}S) labeled mRNA in situ probes and hybridized them to spinal cord tissues that were sectioned longitudinally at 10 μm. To visualize bound riboprobes, tissue slides were dipped in autoradiography emulsion, developed, and counterstained with Nissl stain (Detailed in situ methods published in Burmeister et al., 2008. See also supplementary materials). Using anatomical markers, we identified cells in the lateral motor field of the ventral horn, which contains both motoneurons and interneurons,

[2] The taxonomic assignment of North American leopard frogs is controversial. Both *Rana pipiens* and *Lithobates pipiens* have been used as the scientific name of this species. Publications that we cite in this article refer to this species as *Rana pipiens*, so that is what we use. However, the Amphibian Species of the World taxonomic reference currently recognizes the species as *Lithobates pipiens*.

in both the lumbar and brachial spinal cord of all three frog species. We took photomicrographs of mRNA expression in each motor nucleus, as well as images that reflected background levels of expression (Fig. 2). We quantified the number of silver grains developed in each image of the lateral motor field, then subtracted the number of background silver grains outside of the target area, and divided by all cells in the field of view (See supplementary methods for details). This measure takes into account differences between species in the number of cells in their spinal cords due to differences in body size Larger spinal cords generally have more cells overall, at least in mammals. Similar data is not available for the central nervous system of any frog species (Aitken and Bridger, 1961; Burish et al., 2010). For each spinal cord, we repeated these measurements on at least 4 images of the lumbar cord and the brachial cord taken from separate tissue sections. Our data are therefore representative of AR expression sampled over the entire rostral-caudal and dorsal-ventral extent of the lateral motor field to the extent allowed by the quality of our tissue sections. We tested whether spinal cord AR expression differed among frog species and between spinal cord regions using a linear mixed model with Tukey HSD post hoc comparisons (lme4 package and emmeans package in R, version 4.3.1), accounting for repeated measures by modeling image sample ID nested within individual frog ID as a random effect (Supplementary Methods).

3.2.2. Results

We predicted that AR expression in the spinal cord would differ between species, with *S. parvus* showing higher AR in its lumbar spinal cord compared to the brachial spinal cord. As predicted, we found a significant interaction between species and spinal cord region ($F_{2,\ 111.3} = 5.81$, $P = 0.004$; Fig. 2), suggesting that the pattern of AR distribution across the lumbar and brachial regions is species-specific. However, similar to our previous findings (Mangiamele et al., 2016), there were no overall differences in the level of AR expression in the spinal cord among the frog species ($F_{2,\ 6.68} = 1.95$, $P = 0.21$). At a population level, we noted some differences between species in AR expression levels in the lumbar compared to the brachial spinal cord, although the effects were only marginally significant. *R. pipiens* tended to express more AR in the lumbar spinal cord compared to the brachial (mean difference = 7.28, 95 % CI = 1.96, 12.59, t(80.46) = −2.68, $P = 0.09$), while AR levels in the brachial and lumbar spinal cord of *X. laevis* (mean difference = 6.37, 95 % CI = 0.35, −12.39, t(60) = 2.08, $P = 0.30$) and *S. parvus* (mean difference = 0.64, 95 % CI = −5.78, 7.07, t(50.66) = 0.20, $P > 0.50$) were not significantly different (Fig. 2). In a final analysis, we calculated the ratio of AR expression in lumbar and brachial spinal cord within individual frogs (Table 1). To do this, we averaged AR expression across all lumbar samples from an individual, then divided it by the average AR expression in that individual's brachial samples. Ratio values around 1 reflect equal proportioning of AR between the spinal circuits controlling the hind- and fore limbs in an individual, whereas ratio values >1 mean greater proportioning of AR to the hind limb circuits. Although the ratio values varied a lot among individuals, contrary to our initial prediction, it does not appear that *S. parvus* individuals have consistently higher AR expression in the neurons controlling their hind limbs compared to those that control the fore limb (i.e., they do not have a mean ratio > 1).

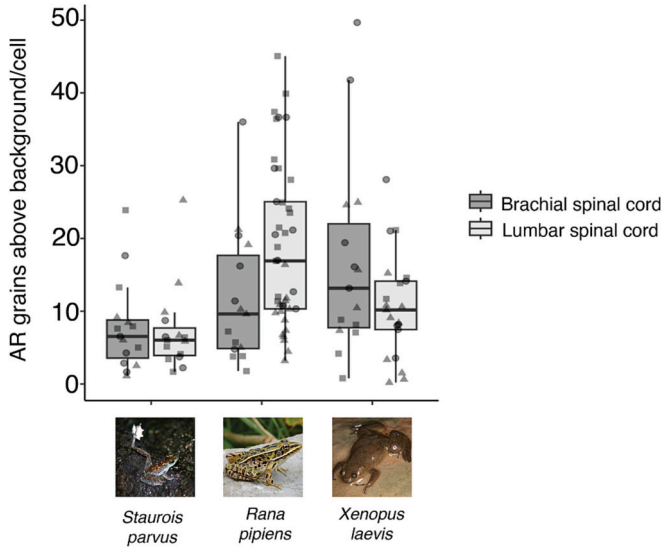

Fig. 2. Comparison of androgen receptor (AR) distribution in the spinal cord of foot-flagging frogs (*Staurois parvus*) and non-foot-flagging frogs (*Rana pipiens, Xenopus laevis*). AR expression is graphed as in situ silver grains above tissue background per cell counted in the field of view. Separate box plots show distribution of AR in the brachial and lumbar spinal cord segments. Points represent measurements of AR expression sampled from individual sections of brachial or lumbar spinal cord in each frog (4–16 sections per frog, $n = 3$ frogs per species). Different point shapes represent different frogs within a species. Photo credits: *Staurois parvus*, Doris Preininger; *Rana pipiens,* Creative Commons/Ryan Hodnett; *Xenopus laevis*, Creative Commons/Brian Gratwicke.

Altogether, these results suggest that there is likely a complex pattern of AR expression distribution in the spinal cord that varies among frog species. For instance, it is possible that patterns of AR expression in the brachial spinal cord may also be explained by other behavioral differences between species, such as differences in the performance of amplexus (clasping) behavior, which is also androgen-mediated (Herrera and Regnier, 1991). Yet, at the same time, contrary to our predictions, we did not find that foot-flagging frogs expressed unusually high levels of AR in the spinal cord cells that control the hind limb compared to non-foot-flagging species. One important caveat of these analyses is that our radioactive in situ does not allow us to distinguish between AR in motoneurons vs. interneurons, as we do not have cellular-level resolution in the localization of AR mRNA. Therefore, possible differences in motoneuron- or interneuron-specific AR expression among species, or between spinal cord regions within a species, are obscured in this data. Follow up studies using newer in situ techniques with cellular-level resolution (e.g., RNAscope) are necessary to evaluate those possibilities.

3.3. Species differences in spinal cord cell density

Despite having no apparent specialization in the androgenic system, we predicted that the hind limb motor circuitry of *S. parvus* was likely to possess at least some unique neurobiological traits that contribute to the ability to produce the unique motor pattern that characterizes foot-flagging behavior. The spinal cord integrates descending inputs from the brain to shape motor output. Therefore, as a first step, we compared

Table 1
Relative AR expression in the brachial and lumbar spinal cord for individuals in our study. Ratios were calculated for each individual frog by dividing the mean AR grains above background/cell in the lumbar spinal cord by the mean AR grains above background/cell in the brachial spinal cord. Ratio values around 1 reflect equal proportioning of AR expression between lumbar and brachial spinal cord within an individual, whereas values >1 reflect greater proportioning to the lumbar spinal cord and values <1 reflect greater proportioning to the brachial spinal cord. Data shown in the last column are mean ratio values ± standard deviation for all individuals of a species.

Species	SubjectID	Ratio AR Expression Lumbar:Brachial	Mean Ratio
Staurois parvus	Sp24	0.39	0.68 ± 0.25
	Sp29	0.84	
	Sp30	0.80	
Rana pipiens	Rp9	5.75	2.56 ± 2.77
	Rp10	1.24	
	Rp11	0.69	
Xenopus laevis	Xl10	2.64	1.16 ± 1.28
	Xl9	0.43	
	Xl12	0.42	

some basic anatomical features of the spinal cord between frog species. We chose to focus on cells in the lateral motor field of the spinal cord's ventral horn, which provide control over the limb muscles. The ventral horn of the vertebrate spinal cord contains two broad categories of cells: motoneurons and interneurons (Fig. 3A). Motoneurons send their axons to skeletal muscles and provide the signal for muscle contraction. Interneurons are important modulators of motor circuits via their connections with multiple motoneurons.

3.3.1. Methods

To assess differences between species in the make-up of spinal motor circuits, we measured the density (i.e., number of cells per 0.089 mm^2 sampling frame) of the large motoneurons that innervate the limbs and their surrounding cells in the lateral motor field – which are presumed to be largely, if not exclusively, interneurons (Ebbesson, 1974; Sotelo and Grofova, 1974) – in sections of the lumbar and brachial spinal cords of *S. parvus*, *R. pipiens*, and *X. laevis* (Fig. 3B). First, we used cytological features to distinguish neurons from non-neuronal cells in Nissl-stained tissue and to exclude any cells that met the criteria of probable glia (García-Cabezas et al., 2016). We then used published anatomical observations in *Xenopus*, *R. pipiens*, and other ranids to identify motoneurons and presumed interneurons and distinguish them from one another using morphological indicators, such as relative size and lateral position within the section (Cruce, 1974; Ebbesson, 1974; Sotelo and Grofova, 1974) (see supplementary methods for additional details). Cells that met these criteria were counted by a trained observer. We then compared

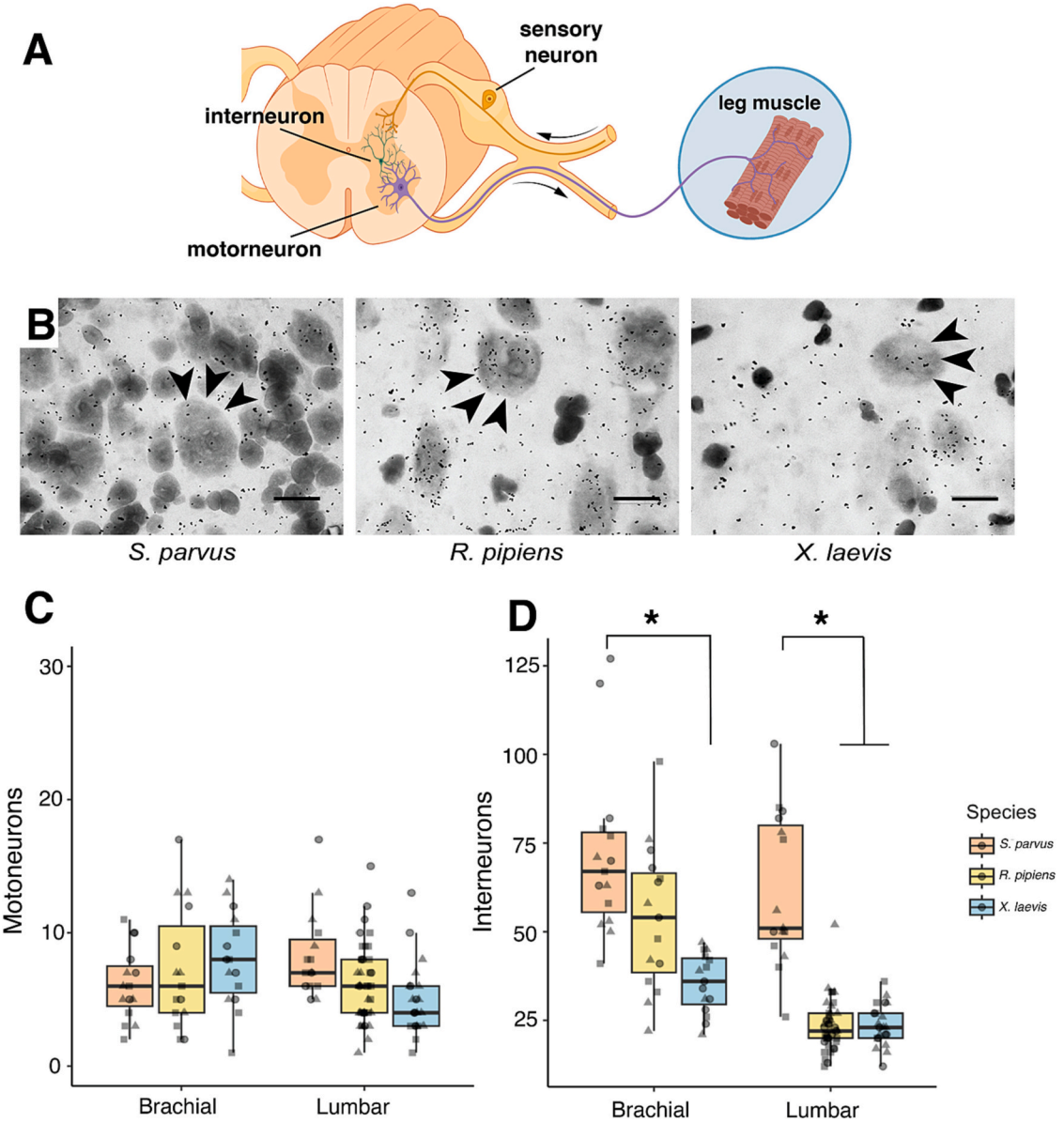

Fig. 3. Species comparison of motoneuron and presumed interneuron cell density in the spinal cord of foot-flagging frogs (*Staurois parvus*) and non-foot-flagging frogs (*Rana pipiens, Xenopus laevis*). (A) Simplified schematic of the neural circuitry in the spinal cord that controls limb movement. Interneurons integrate multiple inputs and project to other cells in the spinal cord, either exciting or inhibiting them. Motoneurons send messages to muscles in the limbs and provide the stimulus for muscle contraction. (B) Representative photomicrographs of lumbar spinal cord tissue sections from in situ hybridization, counterstained with Nissl stain to reveal cell bodies of large motoneurons (arrows) and their surrounding cells, which are presumed to be interneurons. Non-neuronal cells were not counted. Scale bars = 20 μm. (C, D) Plots of the number of motoneurons and presumed interneurons counted in a 0.089 mm^2 image frame. Points represent measurements sampled from individual sections of brachial or lumbar spinal cord in each frog (4–16 sections per frog, n = 3 frogs per species). Different point shapes represent different frogs within a species. Asterisks denote significant post-hoc comparisons ($p < 0.05$). Color figure available online.

species and spinal cord regions using a linear mixed model, as described above.

3.3.2. Results

We found a large difference between species in total neuronal cell density that was opposite of what would be predicted based on body size ($F_{2,\ 5.60} = 12.18$, $P = 0.009$). Foot-flagging frogs had many more spinal cord neurons per sampling frame than either of the larger, non-foot-flagging species. We counted on average 64 % and 105 % more cells in *S. parvus* compared to *R. pipiens* and *X. laevis*, respectively. Interestingly, we found that cell density in the lumbar compared to the brachial spinal cord differed between species (species x spinal cord region: $F_{1,\ 110} = 50.30$, $P < 0.001$). Both *R. pipiens* ($P < 0.0001$, mean difference between spinal cord regions = 31.23 cells, 95 % CI = 20.17, 42.28) and *X. laevis* ($P = 0.01$, mean difference between spinal cord regions = 14.68 cells, 95 % CI = 2.17, 27.20) had many more cells in their brachial spinal cord compared to their lumbar, whereas *S. parvus* had a similar cell density in these two spinal cord regions ($P = 0.07$, mean difference between spinal cord regions = 6.36 cells, 95 % CI = −6.99, 19.72).

When we analyzed motoneurons and presumed interneurons separately, we did not find species differences in overall motoneuron density ($F_{2,\ 6.06} = 0.25$, $P = 0.79$; Fig. 3C), however there was substantial variation in presumed interneuron density among species ($F_{2,\ 5.51} = 12.84$, $P = 0.008$; Fig. 3D) and between spinal cord regions (spinal cord region: $F_{1,\ 110} = 42.84$, $P < 0.0001$; species x spinal cord: $F_{2,\ 110} = 7.72$, $P = 0.0007$; Fig. 3D). In the lumbar spinal cord, the foot-flagging frog, *S. parvus,* had significantly more presumed interneurons than *R. pipiens* ($P < 0.0001$, mean difference between species = 39.18 cells, 95 % CI = 15.93, 62.44) and *X. laevis* ($P = 0.0001$, mean difference between species = 38.64 cells, 95 % CI = 14.58, 62.68). In the brachial spinal cord, again *S. parvus* had more presumed interneurons than *X. laevis* ($P = 0.0006$, mean difference between species = 36.07 cells, 95 % CI = 11.58, 60.55). However, interneuron density was similar between *S. parvus* and *R. pipiens* ($P = 0.29$, mean difference between species = 17.66 cells, 95 % CI = −6.82, 42.15). Within *S. parvus,* interneuron density was equally high in the brachial and the lumbar spinal cord ($P = 0.45$, mean difference between spinal cord regions = 8.74 cells, 95 % CI = −5.18, 22.67). Interestingly, *R. pipiens* frogs had more presumed interneurons in their brachial spinal cord compared to the lumbar ($P < 0.0001$, mean difference between spinal cord regions = 30.26 cells, 95%CI = 18.73, 41.78). These results point to possible anatomical differences between species, specifically in the lumbar spinal cord, that may be associated with the evolution of a novel hind limb signal in foot-flagging frogs. Taken together, our findings suggest that although the androgen sensitivity of the hind limb spinal motor circuit appears similar to that in other species, there may be changes in its anatomy and, possibly, connectivity that could underlie the ability to produce the unique motor patterns of the foot flag.

4. Summary

Uncovering the physiological mechanisms that underlie diversity in communication signals remains a challenging task, however, work in new, non-model systems is helping to reveal fresh insight into the mechanisms that underlie behavioral variation. Here, we have reviewed some of the well-known hormonal mechanisms associated with the production of sex- and species-specific vocalizations in *Xenopus* frogs and compared them with the distinct neuroendocrine phenotype associated with the evolution of a novel hind limb signal in foot-flagging frogs. In doing so, we note some similarities and differences in evolved patterns of tissue-specific androgen sensitivity and cell density that will help us formulate new hypotheses about how the evolution of male signals may unfold in different species (Fig. 4).

From work in *Xenopus* and many other species of anurans, it is clear that androgens are essential to the production of male courtship vocalizations. We have demonstrated that foot flagging, a more recently evolved sexual signal in frogs, is also androgen dependent (Mangiamele et al., 2016). One unique finding that has emerged from our work is a common pattern of increased AR expression in the hind limb musculature in several frog species that have convergently evolved foot flagging,

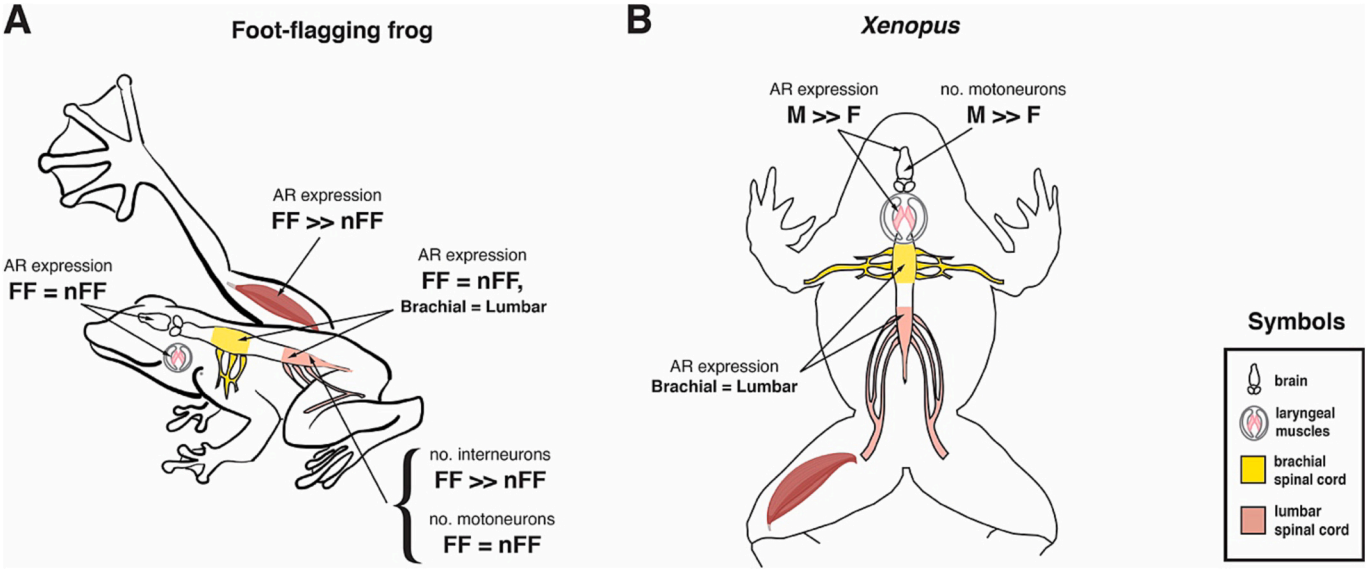

Fig. 4. Comparison of patterns of androgen receptor (AR) expression and cell density in the neuromotor systems associated with signaling behaviors in *Xenopus* and foot-flagging frogs. (A) Species differences in androgen sensitivity have been observed only in the hind limb musculature. Foot-flagging frogs (FF) have much more AR expression in the leg muscle compared to non-foot-flagging frogs (nFF). Species differences in overall AR expression level have not been observed in the larynx or brain. In the spinal cord, neural circuits controlling the fore limbs and hind limbs appear to be equally androgen-sensitive. *Staurois parvus* is unique among the frogs we have studied in having a very high density of presumed interneurons in the lumbar spinal cord. There is not a clear difference between species in the number of motoneurons in the lumbar spinal cord. (B) Sex differences in androgen sensitivity have been observed in the brain and larynx of *Xenopus,* in which males (M) that make courtship vocalizations have higher AR expression than females (F), which do not. Males have more motoneurons controlling their larynx than females do. AR expression is also present in the spinal cord, however, neural circuits controlling the fore limbs and hind limbs appear equally androgen sensitive. Color figure available online.

compared to frogs that have not evolved this behavior (Fig. 4A; Anderson et al., 2021b; Mangiamele et al., 2016). We have now observed this pattern in species of three unrelated families of foot-flagging frogs (Ranidae, Micrixalidae, Hylidae). These data support the idea that when selection favors the production of more complex signaling behaviors, it may also favor androgen-driven muscular changes that allow males to produce novel motor patterns (Fusani et al., 2014; Fuxjager et al., 2015, 2017; Fuxjager and Schlinger, 2015; Regnier and Herrera, 1993). A similar theme is observed in *Xenopus*: the evolution of complex, trilled vocalizations in males, but not females, is associated with large sex differences in androgen sensitivity in the laryngeal muscles, and is a common feature of the *Xenopus* phylogeny. Whether the production of foot flags relies on androgen-driven alterations in fiber type, physiology, or contractile properties in hind limb muscles – like those changes in the larynx required for production of trilled courtship vocalizations in adult male *X. laevis* – is not yet known; however, there is good evidence that androgens do influence the contractile kinetics of frog limb muscles (Regnier and Herrera, 1993). Nevertheless, it clear that heightened androgenic action in the peripheral muscles underlying sexual signal production is an evolutionary path that recurs multiple times in the frog lineage.

However, a more nuanced look at instances of parallel evolution in male *Xenopus* vocalizations shows that there is not a consistent pattern in the mechanisms that underlie variation in signaling behavior between species. For example, in two species (*X. borealis, X. boumbaensis*) that produce vocal patterns that are more simplified than the rapid, trill-like courtship calls of *X. laevis*, the evolution of similar behaviors has proceeded through different mechanisms. The loss of androgen-driven development of fast-twitch laryngeal muscle fibers appears to underlie the simplification of *X. borealis'* calls, while the same is not true for *X. boumbaensis*. In that species, the proportion of fast-twitch fibers is conserved and is more similar to that of *X. laevis* males, which produce much more rapid and complex vocalizations (Leininger and Kelley, 2013, 2015). Thus, in *Xenopus*, the evolution of diversity in the communication signals of different species is not always associated with AR's effects on the larynx.

Why the evolution of male vocalizations in *Xenopus* proceeds along several different routes, while the emergence of foot flagging in several frog lineages seems to share a common mechanism is unclear, however, we might consider the different constraints that may be imposed on the two neuromotor systems in question. As we have previously argued (Mangiamele and Fuxjager, 2018), evolutionary processes may operate differently in circuits used only for sexual communication compared to circuits that animals use for signaling but that have primarily evolved for other purposes (e.g., locomotion). Neuromotor systems that are specialized for vocalization, like the *Xenopus* larynx, may have more evolutionary lability. In particular, the *Xenopus* genus is somewhat unique among anurans; they are aquatic species in which the motor circuits that drive vocalization are not the same ones that drive respiration. Therefore, unlike the hind limb motor circuit, evolutionary changes in the vocal motor circuit are less likely to affect other functions. One hypothesis is that multifunctional circuits have more evolutionary constraints, given that they must be subject to strong natural selection to preserve vital functions, and therefore there are fewer paths selection can take to modify the system without too much perturbation (Katz, 2011; Katz and Harris-Warrick, 1999). With respect to foot-flagging frogs, perhaps increases in muscular AR enables changes to the hind limb neuromuscular system that are the least disruptive and thus can be selected for repeatedly. Alternatively, evolutionary processes might instead shape signals randomly – in other words, lineage-specific mechanisms may arise simply due to genetic drift or to species differences in the level of natural variation in the mechanisms underlying the production of signals (Rosvall, 2022). Interestingly, a few foot-flagging frog species appear to have increased standing variation in hind limb muscular AR expression compared to their non-foot-flagging relatives, which points to this as one possible route through which androgen-mediated gestural displays could have evolved (Anderson et al., 2021b). To further explain this evolutionary pattern, it will be important to understand how developmental and molecular changes affect form and function of the hind limb neuromuscular system in frogs that have evolved the foot flag and how commonly these occur across a broad phylogeny.

In comparing evolutionary patterns of androgen sensitivity, we noted a difference between *Xenopus* and foot-flagging frogs in ***where*** sex and species differences tend to emerge (Fig. 4). As we review above, studies in *Xenopus* demonstrate that androgens influence multiple levels of the vocal motor system to generate diversity in vocal signals. Robust sex differences in androgen receptor expression in the brain, laryngeal nerve, and laryngeal muscles (Fig. 4B) are linked to often dramatic changes in the structure and function of those components that often (but not always) map on to sexually dimorphic vocal features. In addition, recent evidence suggests that more subtle differences in the degree of sexual differentiation in laryngeal function between species may also be related to variation in androgenic action on those tissues (South et al., 2021), although these may include more minor changes to the brain and/or larynx. Thus, the diverse array of androgenic effects on the nervous system and on muscular function in *Xenopus* provides many candidate mechanisms for supporting vocal evolution. By contrast, our work has contributed the idea that in some species, like foot-flagging frogs, signal evolution may be associated with the evolution of androgenic sensitivity in peripheral muscles independently from the central nervous system. Using multiple methodologies, we have repeatedly found that androgen sensitivity in the brain and/or spinal cord is similar in foot-flagging frogs compared to non-foot-flagging species, even when examining AR in specific cell populations of the lumbar spinal cord that are presumably involved in foot flagging.

In foot-flagging frogs, the traits that we have so far found to differ most dramatically from non-foot-flagging species are the level of AR measured in the hind limb muscle (~10× higher in *S. parvus*; Mangiamele et al., 2016) and the number of presumed interneurons in the lumbar but not the brachial spinal cord (Fig. 4A). Considered together, these data lead to the hypothesis that steroid signaling originating from the hind limb muscles might influence the organization of spinal circuits in a way that could support the ability to exert fine control over the hind limb musculature to produce the foot flag. Such a mechanism would also depend on the evolution of species differences in other neuromodulators, such as neurotrophic factors and/or their receptors, to mediate some of the effects of androgens on the development, anatomy, and physiology of the spinal cord. Indeed, retrograde signaling of these molecules from peripheral muscles has been demonstrated to influence the structure and function of spinal motor circuitry in rodents. Activation of muscular AR induces the expression of neurotrophic factors, such as brain-derived neurotrophic factor (BDNF) and nerve growth factor (NGF), which in turn travel retrogradely to the spinal cord to affect the morphology and connectivity of innervating motoneurons, interneurons, and glia (Harrington and Ginty, 2013; Kemp et al., 2011; Ottem et al., 2013; Rand and Breedlove, 1995; Verhovshek et al., 2010; Verhovshek and Sengelaub, 2013). To test our hypothesis in foot-flagging frogs, we could selectively block the actions of AR in the hind limb muscle of adult frogs and/or during development of the hind limbs and measure how that affects both spinal circuits and behavior. This approach is particularly tractable in foot-flagging frogs, given that foot flagging can be observed shortly after metamorphosis to a terrestrial lifestyle (~100 days; Anderson et al., 2022; Preininger et al., 2012), whereas in other systems, it takes much longer for male sexual signals to mature.

In addition, if there are differences in spinal interneuron number between species, it may be particularly important for explaining differences in hind limb motor behavior among frogs. For one, experimental work in several species – including in frogs – has demonstrated an important role of spinal interneurons in generating complex movements by linking together neural circuits that underlie different "motor

primitives," which are the simplified component movements that are the fundamental building blocks of motion (Giszter, 2015; Hart and Giszter, 2010; Levine et al., 2014; Saltiel et al., 2001). As we have described, the foot flag is comprised of five different movement phases, at least some of which can be modulated by T (Anderson et al., 2021a). One possibility is that evolutionary changes in the number of interneurons in the hind limb motor circuit in the foot-flagging frog supports the linking together of these multiple kinematic elements of the foot flag into a unified movement – perhaps one that is derived from other, more evolutionarily conserved movements of the hind limb (e.g., jumping, kicking). Although follow-up studies with cell type-specific markers are necessary to confirm their identity, the comparatively high density of presumed spinal interneurons that we observed in *S. parvus* may also reflect an expansion and/or diversification of cell types, a well-established mode of neural circuit evolution (reviewed in Katz and Harris-Warrick, 1999; Tosches, 2017). In vertebrates, different classes and subpopulations of interneurons in the ventral horn of the lumbar spinal cord are involved in different types of movements; for example, in mammals V1 interneurons influence motoneurons innervating limb flexor muscles, while the V2 class of interneurons are part of the circuitry regulating limb extension (Bikoff et al., 2016; Britz et al., 2015; also reviewed in Zholudeva et al., 2021). A future goal is to further characterize spinal interneurons in *S. parvus* via transcriptional profiling and to use comparative genomics to explore differences between frog species in the expressed genes. As other model organisms have revealed, even small genetic changes can lead to species and strain differences in motor behaviors. For example, a genetic mutation in the Dmrt3 gene, which plays a role spinal cord interneuron specification, has been demonstrated to underlie modified gaits in mice and in horses bred specifically for their unique trotting (Andersson et al., 2012; Bellardita and Kiehn, 2015). In short, future work to unravel variation in spinal motor systems between foot-flagging and non-foot-flagging frogs has the potential to reveal new mechanisms in the evolution of novel behavioral repertoires.

In conclusion, we hope we have highlighted, as others have also done, that research in non-model species is a powerful way to reveal novel mechanisms of behavior, as well as common evolutionary themes, as our field attempts to parse the role of endocrine mechanisms in behavioral evolution and whether and how certain mechanisms are repeatedly used (See also reviews by Arendt and Reznick, 2008; Demas et al., 2007; Rosvall, 2022). Here, we have reviewed research on foot-flagging frogs, a group of species in which we have uncovered some of the neuroendocrine mechanisms associated with the evolution of a novel, recently evolved communication signal. Comparing this work to prior work on the evolution of *Xenopus* vocalizations helps us see whether patterns of evolution in hormone sensitivity and/or the anatomical properties of neural circuitry for signaling behaviors are generalizable vs. species specific. Of course, classic model organisms, such as *Xenopus laevis*, have offered us much in the way of understanding the molecular, neurophysiological, and genetic basis of behavior. Going forward, the increasing availability of experimental tools that can be applied to non-model organisms, such as foot-flagging frogs, will undoubtedly provide more opportunities to address mechanistic questions about how communication behavior evolves.

Data availability

Data and code are available at the Dryad digital repository: https://doi.org/10.5061/dryad.w9ghx3fvd

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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.2024.105502.

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