Increased androgenic sensitivity in the hind limb muscular system marks the evolution of a derived gestural display

Lisa A. Mangiamele[a,1], Matthew J. Fuxjager[b,1,2], Eric R. Schuppe[b], Rebecca S. Taylor[a], Walter Hödl[c], and Doris Preininger[c,d]

[a]Department of Biological Sciences, Smith College, Northampton, MA 01063; [b]Department of Biology, Wake Forest University, Winston-Salem, NC 27109; [c]Department of Integrative Zoology, University of Vienna, A-1090 Vienna, Austria; and [d]Vienna Zoo, A-1130 Vienna, Austria

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Physical gestures are prominent features of many species' multimodal displays, yet how evolution incorporates body and leg movements into animal signaling repertoires is unclear. Androgenic hormones modulate the production of reproductive signals and sexual motor skills in many vertebrates; therefore, one possibility is that selection for physical signals drives the evolution of androgenic sensitivity in select neuromotor pathways. We examined this issue in the Bornean rock frog (*Staurois parvus,* family: Ranidae). Males court females and compete with rivals by performing both vocalizations and hind limb gestural signals, called "foot flags." Foot flagging is a derived display that emerged in the ranids after vocal signaling. Here, we show that administration of testosterone (T) increases foot flagging behavior under seminatural conditions. Moreover, using quantitative PCR, we also find that adult male *S. parvus* maintain a unique androgenic phenotype, in which androgen receptor (AR) in the hind limb musculature is expressed at levels ∼10× greater than in two other anuran species, which do not produce foot flags (*Rana pipiens* and *Xenopus laevis*). Finally, because males of all three of these species solicit mates with calls, we accordingly detect no differences in AR expression in the vocal apparatus (larynx) among taxa. The results show that foot flagging is an androgen-dependent gestural signal, and its emergence is associated with increased androgenic sensitivity within the hind limb musculature. Selection for this novel gestural signal may therefore drive the evolution of increased AR expression in key muscles that control signal production to support adaptive motor performance.

androgen receptor | testosterone | courtship behavior | signal evolution | frogs

Signal evolution in animals has resulted in a variety of complex acoustic and visual displays (1), many of which involve unusual and elaborate physical performances (2–5). The emergence of physical (gestural) displays typically requires adaptation of select motor systems that precisely control how the body and limbs are moved (6–10). However, little is known about the ways in which the neuromotor systems underlying signal production are modified by evolution to help incorporate novel kinematic routines into adaptive display repertoires (11, 12).

Steroid hormone action provides an avenue through which gestural displays may evolve. Androgens, for example, mediate sexual signaling behavior in diverse male vertebrates (13), and one of the primary targets of androgenic action is the musculoskeletal system (14–18). This work has lead researchers to speculate that selection for ritualized mating displays or movement patterns necessary for copulation are supported by a concomitant evolutionary change in the way that androgens influence skeletal muscles (11, 14, 19). Still, this idea has not been tested from a phylogenetic perspective, which requires tracking whether the gain or loss of a gestural signal is marked by a corresponding gain or loss in the muscular androgenic phenotype (20, 21). Without such information, it remains unclear whether the emergence of new signals is associated with adaptive changes in the effects of androgen on the muscles that generate male displays, or whether the observed relationships between male sexual signaling and muscular androgen sensitivity result from other evolutionary processes.

We address this issue in the Bornean rock frog (*Staurois parvus*), which lives near fast-moving streams in the rainforests of Borneo. Like most frogs, *S. parvus* males use acoustic signals for intraspecific communication in reproductive contexts (22, 23). However, in addition to vocalizations, male *S. parvus* signal using highly conspicuous hind leg movements, known as "foot flags" (22–24). This behavior is performed by fully extending a hind limb above the head, rotating it backward in an arc to expose white-colored foot webbing, and then retracting the leg back to the body (Fig. 1 and Movie S1; refs. 22 and 23). Prior studies suggest that *S. parvus* use calls as longer-range advertisement signals, whereas they use foot flags predominantly as close-range signals to other males in competition for breeding sites (22, 25). Thus, in this species, acoustic and visual displays are used in concert as a multimodal signal, or at least a simultaneous and/or sequential combination of signals necessary for appropriate intraspecific communication (22, 26). Importantly, only a few frog

Significance

Diverse species signal using limb gestures, but little is known about how selection incorporates such movements into display routines. We study this issue in a tropical frog that produces complex waving displays with its hind limbs. We find not only that androgenic hormones activate such signaling behavior, but also that the signal's recent evolution is marked by a dramatic increase in androgenic sensitivity of the thigh muscles that control hind limb maneuvering. Moreover, we demonstrate that this muscular phenotype mirrors that which is found in the larynx of other frogs that primarily produce androgen-dependent vocalizations as social signals. We therefore uncover strong coevolution between the emergence of complex sexual gestural signals and enhanced androgenic signaling mechanisms in the muscular system.

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[1]L.A.M. and M.J.F. contributed equally to this work.

[2]To whom correspondence should be addressed. Email: mfoxhunter@gmail.com.

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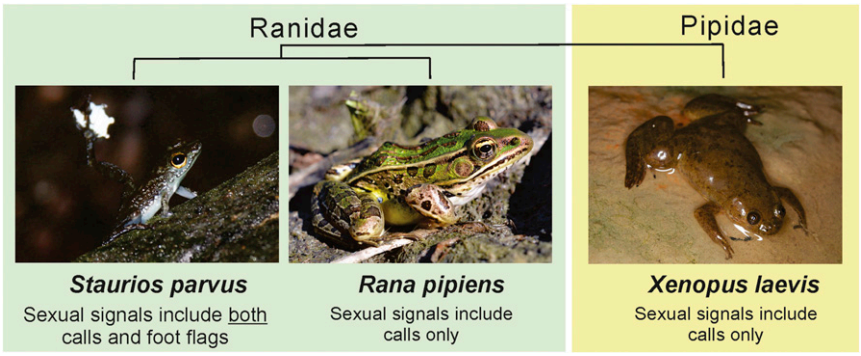

Fig. 1. Phylogenetic relationship among *S. parvus*, *R. pipiens*, and *X. laevis*. The family to which each species belongs is indicated above the phylogeny, and a brief description of the species' sexual signals is indicated below the scientific names. *S. parvus* image courtesy of Doris Preininger; *R. pipiens* image courtesy of Flickr/Andrew C; *X. laevis* image courtesy of Flickr/Brian Gratwicke.

species in the large family (Ranidae) to which *S. parvus* belongs have evolved foot-flagging behavior, which means that it is a derived signal that emerged well after vocal signaling (23). This feature makes *S. parvus* an excellent species to test whether a highly derived gestural signal—foot flagging—is associated with a change in the androgenic phenotype of the muscles that control hind leg kinematics. To date, however, nothing is known about the physiological mechanisms involved with foot flagging.

We hypothesize that foot flagging in *S. parvus* is androgen-dependent and has coevolved with increased androgen sensitivity in the thigh muscles that control femoral extension, rotation, and retraction (27). This idea is rooted in work demonstrating that androgens mediate sexual signaling in frogs (28–30), in large part by acting on peripheral structures, such as the larynx to modulate vocal performance (31, 32). Here, we test this idea in two ways. First, we experimentally manipulate levels of the androgenic hormone testosterone (T) in a captive population of *S. parvus* and measure whether this treatment increases foot-flagging behavior. Second, we use quantitative PCR (qPCR) to measure levels of androgen receptor (AR) mRNA expression in the leg musculature, larynx, whole spinal cord, and whole brain of three anuran species: *S. parvus*, *Rana pipiens*, and *Xenopus laevis* (Fig. 1). We selected *R. pipiens* because it is in the same family as *S. parvus* (33), but does not perform foot flags (23). We selected *X. laevis* because it also does not perform foot flags, and it is a commonly used model to understand endocrine regulation of anuran signaling and its evolution (28, 34). Furthermore, past work shows that androgens modulate sexually selected vocal signals in both of these species (29, 31, 32).

We predict that T treatment will increase foot-flagging behavior in *S. parvus* when two males are placed in close competition with each other. We also expect that *S. parvus* will express more AR in its leg muscles, compared with the other species, given that increased androgenic sensitivity in the thigh muscle may have evolved to support sexual selection of foot flagging behavior.

Results

Behavior. Male *S. parvus* treated with T showed significantly more instances of foot-flagging behavior, compared with males treated with saline vehicle (Fig. 2*A*; $z = 2.12$, df = 128, $P = 0.03$). We found no evidence of an effect of time after T injection on foot-flagging behavior ($z = -0.75$, df = 128, $P = 0.45$) and no evidence of a time × treatment interaction ($z = -0.46$, df = 128, $P = 0.64$). As predicted, vocalizations were not the predominant signal produced by males placed in close range to each other. We observed no instances of calling behavior in males injected with saline, and we found only a few instances of calling behavior in males injected with T (mean ± SEM = 0.80 ± 0.33; Fig. 2*B*).

Finally, activity levels measured in frogs from both treatment groups were statistically indistinguishable ($t_{17} = 0.92$, $P = 0.37$). This result is consistent with the idea that T injections neither affected the animals' general locomotory behavior, nor adversely affected their health.

AR Expression Patterns. Overall, we found striking variation in AR expression profiles across species (Fig. 3*A*; $F_{2,13.7} = 12.06$, $P = 0.001$) and among tissues (Fig. 3*B*; $F_{3,33.1} = 9.60$, $P < 0.001$). On average, *S. parvus* expressed more AR in the tissues we examined than either *R. pipiens* ($P = 0.004$) or *X. laevis* ($P = 0.001$), both of which expressed levels of AR that were statistically indistinguishable ($P = 1.0$). When looking across tissues, the larynx expressed the highest amount of AR, compared with the leg muscle ($P = 0.001$) and brain ($P = 0.006$).

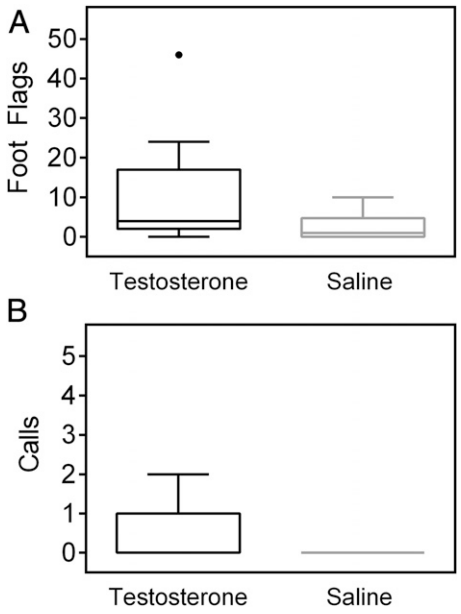

Fig. 2. Testosterone (T) increases the number of foot flags (*A*), but does not appear to affect the number of calls (*B*), performed by interacting male *S. parvus*. Horizontal lines indicate median values. Upper and lower boundaries of the boxes denote upper and lower quartiles and error bars denote maximum and minimum values ($n = 10$ interactions per treatment group). Note that few calls were produced in the experiment; this result was expected because our experimental setup forced relatively close-range communication, which is primarily accomplished through foot flagging in *S. parvus* (22). Also note the different vertical axis scales on *A* and *B*.

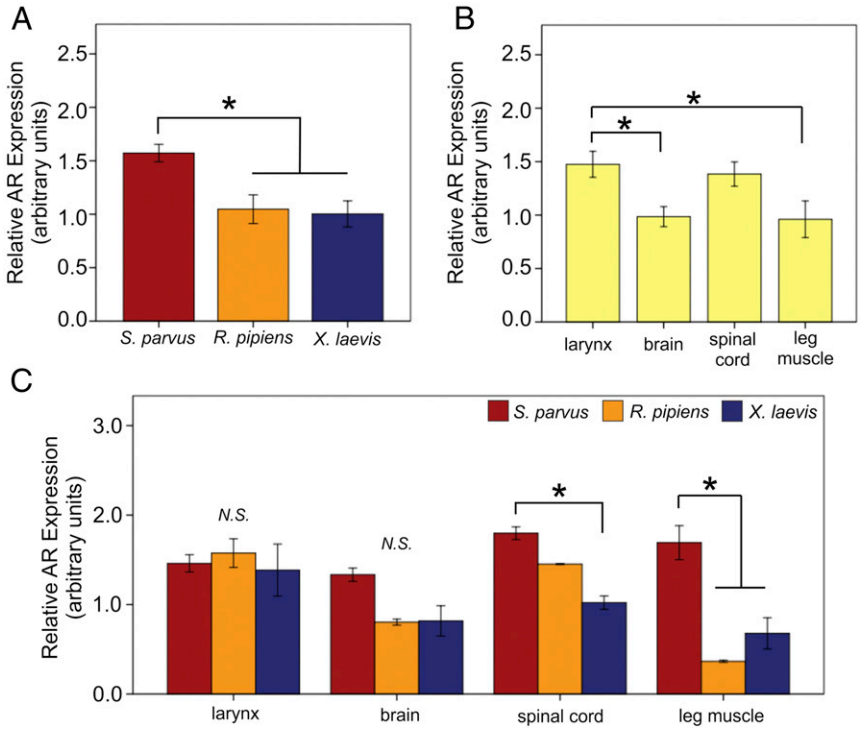

Fig. 3. Androgen receptor (AR) expression level varies between species (*A*) and tissues (*B*) examined. Comparison of AR expression between species within a tissue type (*C*) shows a dramatic increase in AR in spinal cord and leg muscle of *S. parvus*. Asterisks indicate significant differences between species or tissues (*$P < 0.05$; N.S., not significant). Data represent means ± SEM.

Most of the variation in AR expression was tissue-specific (Fig. 3*C*; $F_{6,32.7} = 3.91$, $P = 0.005$). As we predicted, levels of AR expression were higher in the leg muscles of *S. parvus*, relative to leg muscles of both *R. pipiens* ($P < 0.001$) and *X. laevis* ($P < 0.001$). In the spinal cord, *S. parvus* had similarly high AR levels, which were greater than *X. laevis* ($P = 0.017$), but not *R. pipiens* ($P = 0.62$). Interestingly, there was no species difference in laryngeal AR ($P = 1.0$) and brain AR ($P \geq 0.10$).

We also found substantial variation in AR expression among tissues within a species. In *R. pipiens*, for example, individuals expressed more AR in their larynx, compared with their brain ($P = 0.011$) and leg muscle ($P < 0.001$). In *R. pipiens*, spinal cord AR was also greater than leg muscle AR ($P = 0.002$). This pattern of expression was comparable to *X. laevis*, in which males expressed more AR in their larynx, compared with their leg muscle ($P = 0.012$). Interestingly, there was no difference in the amount of AR expression across tissues in *S. parvus* ($P \geq 0.58$).

Relative AR Expression in the Leg Muscle and Larynx. In a subset of individuals within our study, we examined the ratios of AR expression in the leg muscle to larynx (Fig. 4). For this analysis, ratio values around 1 reflect an equal proportioning of AR between leg muscle and laryngeal tissues within an individual. By contrast, ratio values closer to 0 reflect greater proportioning of AR in the larynx, compared with the leg muscle. Our analysis shows a significant difference in this ratio across species ($F_{2,11} = 7.27$, $P = 0.011$). Post hoc analyses demonstrate that the ratio of leg muscle:larynx AR is significantly greater in *S. parvus*, compared with both *R. pipiens* ($P = 0.012$) and *X. laevis* ($P = 0.038$). Furthermore, because the average ratio value in *S. parvus* is approximately 1, the data indicate that individuals of this species maintain roughly equal proportions of AR expression in their leg musculature and their larynx, which are both used in the animal's signaling repertoire. The lower ratios in *R. pipiens* and *X. laevis* indicate that these species, which use only vocal signals, maintain relatively more AR in their larynx compared with their leg muscles.

Discussion

Our results demonstrate that the evolutionary gain of a novel sexual signal is associated with a dramatic change in the androgenic phenotype of the skeletal musculature that actuates signal production. Our work centers on *S. parvus*, which is a tropical frog from the family Ranidae that has recently evolved the ability to produce foot flags (Fig. 1) to augment close-range sexual communication in noisy environments (23). Compared with two species that do not perform foot flags (*R. pipiens* and *X. laevis*), we found that adult male *S. parvus* express ~10× more AR in the main thigh muscles that mediate femoral rotation, flexion, and extension (27), which are the movements that make up the foot flag (23). Thus, our results strongly indicate coevolution of the foot flag and the level of androgenic sensitivity in the musculature controlling the signal.

We also show that exogenous administration of T increases foot-flagging behavior in *S. parvus* under seminatural conditions,

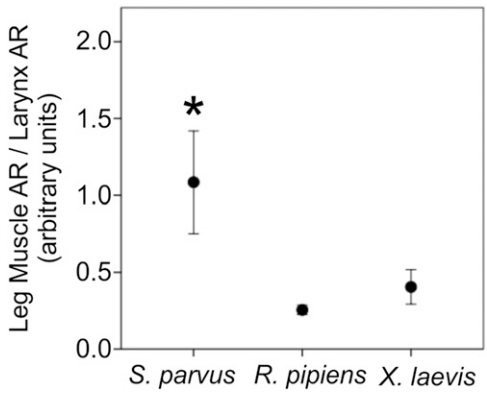

Fig. 4. Ratio of AR expression in leg muscle to larynx in individuals of all three species. Asterisk indicates that *S. parvus* has a much higher ratio compared with either of the other two species (for post hoc tests, see *Materials and Methods, Data Analysis*). Data represent means ± SEM.

indicating that, like frog vocalizations (29, 30), foot flags are an androgen-dependent signal. At the same time, T administration failed to influence either general locomotion or calling behavior. Thus, we can rule out the possibility that T influenced foot flagging by having nonspecific effects on neuromotor systems, such as by altering general activity levels. Rather, we conclude that T impacts foot flagging behavior in part by acting on AR populations in the thigh muscle that likely influence foot flag kinematics. With respect to calling behavior, we recognize that the lack of any effect of T is at first counterintuitive. However, this finding aligns well with our understanding of signaling behavior in *Staurois*, because males primarily use calls as long-distance signals that alert the receiver to the subsequent foot flag (22, 25). Because males in our experimental setup were forced to interact with each other at close range (within a few centimeters), we would not expect them to broadcast calls to each other.

Finally, our data reveal that the relative proportion of AR expressed by an individual in its leg musculature and larynx corresponds to the type of displays in a species' signaling repertoire. *S. parvus* individuals showed equal portioning of AR in both the leg muscle and larynx, which are used in producing multimodal displays. By contrast, individuals of the other two species, which mainly use vocalizations for intrasexual and intersexual signaling, showed far more AR in their larynx than in the thigh muscles. These results therefore imply that the pattern of AR expression in *S. parvus* emerged to support the use of multiple signals in sexual communication.

To our knowledge, ours is the first study to demonstrate that the evolutionary gain of a sexual signal within a clade is marked by the emergence of a novel pattern of sex steroid receptor expression in the muscles that effectuate behavioral output. Previous studies have proposed this idea (11, 14), whereas other work has shown that steroid hormones, such as androgens, can act on muscle to influence adaptive behavioral performance (35). However, such work has not been placed in a phylogenetic context to show that a signal's emergence (or loss) is linked with a novel target for sex steroids. The closest report of such a relationship is in manakin birds, showing that species variation in physical display complexity is positively correlated with interspecific variation in muscle AR levels (14). Yet this study does not identify whether the origination of the physical display is accompanied by a marked change in androgen sensitivity. Other work has shown that the evolution of sexual dimorphisms in signaling traits is associated with differences in androgen sensitivity of the associated musculature (e.g., frog larynx, frog forelimb clasping muscles; refs. 36 and 37); however, this type of comparison conflates the issue of signal evolution with the endocrine mechanisms that enable sexual differentiation, and it does not directly address the question of how neuromotor substrates are modified by sexual selection to generate new male signals when others previously exist. Therefore, our study offers the first support of the hypothesis, to our knowledge, that sexual signals and androgen sensitivity coevolve, and we speculate that it is applicable to a diverse array of vertebrate taxa that incorporate displays of motor skills into their sexual signaling repertoires (38, 39).

There are two evolutionary scenarios that potentially explain the relationship we observed between foot signaling and thigh muscle AR. Selection for the foot flag display may drive the evolution of differences in AR expression profiles to support foot flag production, or selection for a specific AR expression profile might enable the evolution of the gestural display. Our current data do not distinguish between these two possibilities; however, given our understanding of the factors thought to promote the emergence of the foot flag, we suspect the former model is the most likely to have occurred. Indeed, it is thought that the foot flag evolved as a ritualization of an aggressive leg-kick, given that this display is commonly performed when males are competing for access to signaling sites, resources, and/or mates (40). Additional ecological factors are also considered to have fine-tuned the signal, because it has appeared almost exclusively in frog species that breed alongside fast-flowing streams (refs. 23 and 41, but see ref. 42), where selection favors conspicuous visual signals that improve communication in noisy environments (43, 44). Thus, we speculate that selection for the foot flag drove the evolution of increased muscular AR to support the complex and otherwise unusual movements necessary to perform this signal.

From a physiological perspective, our study also begs the question: How does elevated muscular AR support the production of the foot flag? There are two possible answers, which are not mutually exclusive. The first is that androgens may modify the hindlimb musculature in a way that augments foot movement or sustains foot flag production. This idea is supported by work in manakin birds that shows that activation of muscular AR locally up-regulates the expression of genes that likely enhance muscle fuel metabolism and contractility, which are necessary to perform the rapid and elaborate wing-snap displays (19, 45). Similarly, in *X. laevis*, androgens are known to increase the number, size, and contractile properties of muscle fibers in the larynx to increase the click rate of vocalizations (36, 46). Thus, a similar effect may occur in *S. parvus*, whereby activation of AR optimizes the transcriptional machinery of the thigh muscles to somehow enhance these tissues' ability to perform the display. Whether these events occur in *S. parvus* is not known, but future work to examine how androgens influence gene expression patterns in the neuromotor pathways underlying foot flagging behavior can test this idea. The second possible answer to how elevated muscular AR supports foot flagging is that activation of such AR helps maintain the neural circuitry within the spinal cord that enables motor control of the hind limb in *S. parvus*. In support of this view, numerous studies in mammals show that the activation of AR populations in specific muscles induces the expression of signaling proteins that travel from the muscle to the spinal cord via the innervating motor neuron. These signals influence motor neuron morphology and connectivity in the spinal cord (47, 48), and blocking such retrograde signaling impairs motor control and performance (49, 50). Thus, elevated levels of AR in the thigh muscles of *S. parvus* may support the spinal architecture that helps control the unique movements that make up the foot flag display.

One aspect of our study that merits further investigation is how AR in the spinal cord is related to the evolution and production of the foot flag. Prior work has speculated that increased expression of spinal AR is important for wing-snap displays in manakins (15, 51), although this hypothesis is not widely explored in other bird species that produce gestural display routines. Our current results do not conclusively demonstrate that foot signaling coevolved with increased AR levels in the spinal cord, because spinal AR expression in foot flagging *S. parvus* was statistically indistinguishable from that in the nonfoot flagging *R. pipiens* (although both of these species express more spinal cord AR than *X. laevis*). These results are consistent with the idea that the total expression of AR in the spinal cord of *S. parvus* is not associated with the emergence of the foot flag per se. However, we caution that there may be other modifications to the androgen sensitivity of the spinal cord that we could not measure using qPCR, including the redistribution of AR within the spinal cord of *S. parvus* to the lumbar spinal motor neurons that control the hind limbs. Testing this idea will require comparison of AR expression within the lumbar region of the cord in *S. parvus* and nonfoot flagging species using techniques, such as in situ hybridization, that allow for precise localization and quantification of AR in specific motor neuron populations.

In summary, our work provides evidence that a derived foot-flagging display in *S. parvus* coevolves with a dramatic increase in AR expression in the thigh muscles that control this signal. These findings provide insight into how the evolutionary gain of a sexual display trait may be augmented by evolution of the hormone systems that control and refine adaptive motor skills. Thus, our work may apply to a host of vertebrate taxa that have similarly evolved unique gestural displays. Nonetheless, our current study emphasizes the need

for future research to further test whether the emergence of gestural displays is explained by concomitant evolution of increased androgenic sensitivity within musculoskeletal systems alone, or in both the muscles and the central nervous structures that control them.

Materials and Methods

Animals. Animals were maintained and the following procedures were approved by the appropriate Institutional Animal Care and Use Committees at Smith College and Wake Forest University, as well as the University of Vienna and the Vienna Zoo, both of which follow the European Union Directive.

Adult male Bornean rock frogs (*S. parvus*) were bred in captivity at the Vienna Zoo, Vienna, Austria, from wild animals captured in 2010 near fast-flowing streams in Ulu Temburong National Park, Brunei Darussalam. They were housed at a temperature (23–25 °C), relative humidity (70–90%), and day length (12 h of light:12 h of dark) that closely approximates conditions in their native Borneo in a large terrarium that houses ∼150 frogs. Under these conditions, the frogs vocalize, perform foot-flagging displays, and breed nearly all year long (52), as they do in the wild.

Adult male *R. pipiens* were obtained from a commercial supplier (eNasco) that breeds and houses the animals in outdoor enclosures. They were captured during their natural breeding season (March), were shipped overnight, and housed in groups in the Smith College Animal Care Facility for 3 wk at 18 °C in terraria with a shallow pool of water. We observed males vocalizing in captivity, and all males had large nuptial pads on their "thumb" digit of the forelimb, a morphological feature that undergoes seasonal growth and is androgen-dependent in *R. pipiens* (53). Adult male captive-bred *X. laevis* were obtained from a commercial supplier (Xenopus Express) in May and were housed together at 20 °C in large aquaria. We observed males clasping females and all males had large, dark nuptial pads on their forelimbs, an indicator of sexual maturity.

Behavioral Testing. Behavioral tests on *S. parvus* (n = 40 total males) were conducted over 10 d, between 1200 and 1900 hours each day. At the onset of each testing day, we captured reproductively active adult males that were observed foot flagging in their home terrarium. We then randomly assigned these individuals to receive a 20-μL s.c. injection of either (*i*) T propionate in saline (dose: 1 μg/g body weight) or (*ii*) saline vehicle only (control). Past studies have used similar doses of T to effectively study anuran endocrinology and behavior (54). Immediately after administering these injections, we placed two males that received the same treatment together in a small arena (16.5 × 12.5 × 12.5 cm) that was encased in transparent mesh. We then randomly chose an adult female from the home terrarium and placed her in the arena alongside the two males. Each arena was subsequently placed in its own larger enclosure (∼60 × 35 × 35 cm) that was designed to mimic the animal's natural breeding environment. Enclosures were lined with Styrofoam and acoustic foam padding, and they all contained a single plant and a source of running water to simulate the presence of a waterfall. To further simulate the natural environment, we played back a 30-s recording of *S. parvus* vocalizations once every 5 min. Overall, this setup mimicked the animals' breeding environment (52), while forcing males into close proximity where they are more likely to produce foot flags (22, 25). The transparent mesh material that covered the arena allowed the frogs to be easily viewed and recorded. In total, we included 10 separate mesh arenas (with 2 males per arena) in each treatment group. No frogs (including both males and females) were used in this study more than once.

Immediately following a 1-h postinjection acclimation period, we videotaped the frogs' behavior for the next 7 h, until lights out (1900). Later, an observer blind to treatment group watched these videos and measured both foot flagging and calling behavior performed by both individuals in the arena. For foot flags, we counted the frequency of this behavior per hour produced by either male. For calls, we counted the number of vocalizations produced by males in an arena by first finding the call's acoustic signature on spectrograms of the video's audio track and then confirming observable movement of an individual's vocal sac at the correct time on the appropriate video. We were unable to distinguish individual males within the same mesh arena, and, thus, behavioral measures are recorded on a per arena basis (with n = 10 arenas per treatment group).

To validate that T treatment did not impair the frogs' movement or general health during the experiment, we assessed levels of physical activity between the treatment groups. We randomly chose 1 h of video between hours 3 and 5 of the behavioral observation session, which corresponds to time of peak afternoon activity (52). We then sampled the video at 30-s intervals, scoring activity in 10-s time blocks. If any frog showed movement during the 10 s, we scored that interval with a 1; if no movement was observed, we scored that interval with a 0. We then averaged the scores for each arena and used this measure as our metric of overall activity.

Tissues Collection. We collected whole brain, whole spinal cord, larynx, and thigh muscle from *S. parvus*, *R. pipiens,* and *X. laevis* (n = 6 individuals per species, with the number of tissues collected per individual varying from 4 to 6 because some tissues were used for another experiment). We killed animals by using rapid decapitation and then quickly dissected out the tissues of interest. Leg muscle samples were composed of all of the muscular tissues that made up the thigh, given that these tissues drive femoral extension, rotation, and retraction movements (27) that collectively make up foot flag kinematics (22, 23). We preserved samples in RNA Later (Invitrogen) according to the manufacturer's instructions and then stored the samples at −80 °C until RNA extraction. All dissections were performed identically across species, and tissues were treated and preserved in an identical manner.

RNA Isolation and Reverse Transcription. We isolated RNA from each sample with TRIzol (Invitrogen) according to the manufacturer's instructions. At the onset of this process, samples were homogenized for 30–40 s at medium speed with a rotor/stator homogenizer. Final concentrations of RNA were determined by using a Nanodrop system (Thermo Scientific), whereas RNA integrity was verified by using gel electrophoresis. Following DNase treatment, we reverse transcribed 1 μg of RNA by using SuperScript II Reverse Transcriptase (Invitrogen). This reaction occurred for 50 min at 42 °C, followed by 15 min at 70 °C. The resulting cDNA was then used for PCR amplification of genes of interest, as well as quantitative real-time PCR assessment of gene transcription levels (see below).

Identification of AR mRNA Sequence in *S. parvus*. To identify AR in *S. parvus* cDNA, we used degenerate primers used previously to amplify AR in túngara frog (22, 23, 26). We then performed PCR by using the following parameters: 25–30 cycles of 95 °C for 30 s, 56–51 °C for 30 s (Δ −0.2/cycle), 72 °C for 3 min, and 15 cycles of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 3 min. We sequenced the fragment and submitted it to GenBank (accession no. KU350627), and we compared our *S. parvus* AR sequence to that of the published AR mRNA sequences of *R. pipiens* and *X. laevis* from GenBank (*R. pipiens*: accession no. EU350950.1; *X. laevis*: accession no. BC170347.1). The degree of similarity between AR in all three species was high (>80% identical), such that *S. parvus* AR is 95% identical to *R. pipiens* AR and 81% identical to *X. laevis* AR.

We used a similar approach to identify the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in *S. parvus*. However, instead of using degenerate primers to amplify *S. parvus* GAPDH, we used primers designed from the published GAPDH *R. pipiens* sequence (GenBank accession no. KJ466336.1; Forward: GGTGTGTTCACAACCACTGAAAA; Reverse: ATGCCAGTGATTTTTCCGTTCAG). PCR cycling parameters were similar to those described above. We submitted *S. parvus* GAPDH sequence to GenBank (accession no. KU350626), and we found that this sequence was highly similar to that of *R. pipiens* (98%) and *X. laevis* (81%; GenBank accession no. NM001087098.1).

qPCR. All reactions were performed in an Applied Biosystems 7500 Fast Real-Time sequence detection system, using SYBR Green Master Mix kits (Applied Biosystems). Each reaction contained 100 ng of template, 0.9 mM forward primer, and 0.9 mM reverse primer. We developed species-specific primers for AR (gene of interest) and GAPDH (control housekeeping gene) with the sequences obtained above (Table S1). Given the high degree of sequence homology between *S. parvus* and *R. pipiens*, we developed a single set of primers that annealed to regions of each gene that were identical between the two species. All reactions were run by using the following parameters: 50 °C for 2 min, 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s, and 60 °C for 1 min. We added a final dissociation stage to the end of the reaction process, which consisted of 95 °C for 15 s, 60 °C for 30 s, and finally 95 °C for 15 s. All reaction efficiencies were between 90 and 100%, and dissociation curves were used to verify the absence of contamination. We ran samples in duplicate, and we used the standard curve method to measure relative expression of AR in each sample (i.e., quantity AR/quantity GAPDH).

Data Analysis. We compared the number of foot flags between T-treated and saline-treated *S. parvus* males by using a zero-inflated Poisson mixed effects model in R (glmmADMB; refs. 55 and 56). We modeled treatment (saline, T), time (hours after injection), and their interaction as fixed effects and male pair ID as a random effect. We chose this model because it allows repeated measurements from the same individuals to be fitted as a random variable, thus better controlling for differences in signaling behavior between pairs of males, while also allowing for overdispersion of zeroes in our counts of behavior over the 7 h of video.

We analyzed differences in overall activity scores between treatment groups by using a Student's t test. This test was selected because activity scores were obtained from individuals during only one randomly chosen hour during the peak times of foot flagging (i.e., between hours 3 and 5 of

the behavioral observation), which meant that there was no within-subjects factor for which we needed to account.

We compared AR gene expression among species by using a linear mixed-model ANOVA in SPSS. Species and tissue were modeled as fixed factors, whereas individual ID was modeled as a random factor. Significant main effects and interactions were assessed by post hoc pairwise comparisons or contrasts, respectively, whereas Bonferroni corrections were used to control for an inflated alpha value (57).

In a final analysis, we examined species differences in the ratios of AR expression in the leg muscle to AR expression in the larynx. Ratio values around 1 reflect an equal proportioning of AR between these two tissues, whereas ratio values closer to 0 reflect greater proportioning of AR in the larynx, compared with the leg muscle. Ratios were compared by using a one-way ANOVA, with significant main effects followed by Bonferroni-corrected post hoc pairwise contrasts.

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