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

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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 ∼10x 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

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.


The authors declare no conflict of interest.

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Data deposition: The sequences reported in this paper have been deposited in the GenBank database (accession nos. KU350627 and KU350626).

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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 anuran 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. 2A; $z = 2.12$, 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 x 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. 2B).

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. 3A; $F_{2,13,7} = 12.06$, $P = 0.001$) and across tissues (Fig. 3B; $F_{3,3,3.1} = 9.60$, $P < 0.001$). 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 ($P = 0.006$).
Most of the variation in AR expression was tissue-specific (Fig. 3C; $F_{6,32.7} = 3.91, P = 0.005$). 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 actsuate 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 ~10x 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. 3](image-url) 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 $\pm$ SEM.

![Fig. 4](image-url) 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 $\pm$ 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 traits in signaling traits is associated with differences in androgen sensitivity of the associated musculature (e.g., frog larynx, frog fore-limb 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 display trait 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 wing-snap 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 footflagging 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 androgen 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 frog 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 light:12 h dark) that closely approximated 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. p. p. p. (40 total males) 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. p. p. (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. i. 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 (~50 × 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 natural environment. We observed males vocalizing in captivity, and they all contained 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. p. p. (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.

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 GAPDH in turgara 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 (a ~0.2/°C), 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. p. p. and X. laevis from GenBank (R. p. p. accession no. EU350950; X. laevis accession no. BC170347). The degree of similarity between AR in all three species was high (>80% identical), such that S. parvus AR is 95% identical to R. p. p. AR and 81% identical to X. laevis AR.

Tissues Collection. We collected whole brain, whole spinal cord, larynx, and thigh muscle from S. parvus, R. p. p., 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 turgara 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 (a ~0.2/°C), 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. p. p. and X. laevis from GenBank (R. p. p. accession no. KJ466336.1; X. laevis accession no. KC409130.1). The degree of similarity between AR in all three species was high (>80% identical), such that S. parvus AR is 95% identical to R. p. p. AR and 81% identical to X. laevis AR.

qPCE. All reactions were performed in an Applied Biosystems 7500 Fast Real-Time sequence detection system, using SYBR Green Master Mix kits (Applied Biosystems) at 100 ng of template DNA, 100 ng of 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. p. p., 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, 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 flag/follows 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 zeros 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
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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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