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RESEARCH ARTICLE

A novel degree of sex difference in laryngeal physiology of *Xenopus muelleri*: behavioral and evolutionary implications

Kelly E. South[1,*], Bernhard Klingenberg[1,2] and Elizabeth C. Leininger[1,‡]

ABSTRACT

Characterizing sex and species differences in muscle physiology can contribute to a better understanding of proximate mechanisms underlying behavioral evolution. In *Xenopus*, the laryngeal muscle's ability to contract rapidly and its electromyogram potentiation allows males to produce calls that are more rapid and intensity-modulated than female calls. Prior comparative studies have shown that some species lacking typical male features of vocalizations sometimes show reduced sex differences in underlying laryngeal physiology. To further understand the evolution of sexually differentiated laryngeal muscle physiology and its role in generating behavior, we investigated sex differences in the laryngeal physiology of *X. muelleri*, a species in which male and female calls are similar in rapidity but different with respect to intensity modulation. We delivered ethologically relevant stimulus patterns to *ex vivo X. muelleri* larynges to investigate their ability to produce various call patterns, and we also delivered stimuli over a broader range of intervals to assess sex differences in muscle tension and electromyogram potentiation. We found a small but statistically significant sex difference in laryngeal electromyogram potentiation that varied depending on the number of stimuli. We also found a small interaction between sex and stimulus interval on muscle tension over an ethologically relevant range of stimulus intervals; male larynges were able to produce similar tensions to female larynges at slightly smaller (11–12 ms) inter-stimulus intervals. These findings are consistent with behavioral observations and present a previously undescribed intermediate sex difference in *Xenopus* laryngeal muscle physiology.

KEY WORDS: Frog, Vocal communication, Evolution, Larynx, Neuromuscular, Sex differences

INTRODUCTION

Sex-specific communication behaviors are diverse in form and degree. Behaviors with highly obvious sexual dimorphisms, such as the vocalizations of *Xenopus laevis* (Tobias and Kelley, 1987) and midshipman fish (McIver et al., 2014), or copulatory behaviors in rodents (Seney et al., 2009), have traditionally been very useful for quantifying sex differences in behavior and elucidating their underlying hormonal and physiological mechanisms. Resolving the evolutionary trajectories of sex-specific behaviors and their mechanisms requires the inclusion of species that may display diverse degrees of sex difference in physiology and behavior (Zhou and Smith, 2006). The broad prioritization of highly dimorphic behaviors over subtler sex differences has left an incomplete picture of how such behaviors develop according to sex and evolve across species.

Variation in behavioral sex differences are sometimes – but not always – reflected in underlying neuromuscular physiology and anatomy. For example, the degree of sex differences in African mole rat genital and perineal muscle morphology correlates with species differences in social structure and mating systems (Seney et al., 2009). In contrast, a study examining dewlap signaling across nine species of anoles found that sex differences in dewlap extension rates during displays were not correlated with underlying features of dewlap muscle anatomy such as muscle length and fiber diameter (Johnson and Wade, 2010). A survey of syringeal muscle across 10 species of songbirds with sexually dimorphic vocal repertoires revealed that most species exhibited sex differences in gross syringeal morphology, but eight of the 10 species had no sex difference in superfast muscle fiber composition within the syrinx (Christensen et al., 2017). These findings suggest that physiological mechanisms cannot necessarily be predicted by behavior alone.

A complicating factor in understanding the evolution of neuromuscular substrates for behavior is that communication signals are typically complex and may rely on multiple physiological mechanisms that map to behavioral features in complex ways. Vocal signals in particular contain spectral, temporal and intensity features that may be controlled by distinct physiological mechanisms within the central or peripheral nervous system and effector organs (Kelley et al., 2020; Ryan and Guerra, 2014). Therefore, systems in which specific physiological mechanisms map to a parameter of the signal – for example, the contractile speed of a muscle as a determinant of the sound pulse rate – can be useful in understanding how specific parameters of a signal can evolve.

In this study, we used *Xenopus* frogs as a system for understanding how laryngeal physiology shapes behavioral sex differences within an evolutionary context. The *Xenopus* larynx is an ideal system for mapping neuromuscular mechanisms to specific vocal parameters. The isolated larynx is physiologically robust for several hours *ex vivo* (Leininger et al., 2015; Potter et al., 2005; Tobias and Kelley, 1987). Stimulation of the laryngeal nerve is sufficient to elicit laryngeal muscle contraction, which separates cartilage discs necessary for sound pulse production (Kwong-Brown et al., 2019). Thus, the *ex vivo* larynx is a simple neuromuscular circuit with robust *ex vivo* activity and straightforward correspondence between physiological mechanisms and behavioral features, described below.

Physiological characteristics of the *Xenopus* larynx, such as the potentiation of the laryngeal electromyogram (EMG) and the laryngeal muscle's ability to contract and relax rapidly, support call features such as intensity modulation and inter-pulse interval, respectively (Fig. S1). The summed electrical activity of the laryngeal muscle in response to nerve stimulation can be measured

[1]Division of Natural Sciences, New College of Florida, Sarasota, FL 34243, USA. [2]Department of Mathematics & Statistics, Williams College, Williamstown, MA 01267, USA.
*Present address: Max Planck Florida Institute for Neuroscience, Jupiter, FL 33458, USA.

‡Author for correspondence (eleininger@ncf.edu)

K.E.S., 0000-0003-0261-8341; B.K., 0000-0001-9959-9989; E.C.L., 0000-0002-5638-5005

Received 19 June 2020; Accepted 22 December 2020

Journal of Experimental Biology (2021) 224, jeb231712. doi:10.1242/jeb.231712

List of abbreviations

EMG	electromyogram
hCG	human chorionic gonadotropin
ICI	inter-call interval
IM	intensity modulation
IPI	inter-pulse interval
ISI	inter-stimulus interval
PI	potentiation index
PTT	percent transient tension

with an EMG; repeated stimulation of the laryngeal nerve can elicit progressive increases in EMG amplitude, depending on the strength of the neuromuscular synapse (Ruel et al., 1997). Calculating the fold-change in EMG amplitude (potentiation index; PI) in response to a series of stimuli allows for a general quantification of the progressive recruitment of muscle fibers. Thus, the PI is a neuromuscular correlate of the ability to produce calls with intensity modulation (IM), the progressive increase in sound pulse amplitude over a call that is characteristic of many male *Xenopus* calls (Tobias and Kelley, 1987; Tobias et al., 2011). Separately, the ability of the laryngeal muscle to contract and relax in response to varying rates of nerve stimulation can be measured via a force transducer placed on the tendon attaching the laryngeal muscle to the sound-producing cartilage discs. From the resulting tension transients, it is possible to calculate the percent of transient tension (PTT), or the extent to which a tension transient returns to baseline (called percent relaxation in more recent literature; Fuxjager et al., 2016; Miles et al., 2018). The laryngeal muscle's ability to sustain high PTT at short inter-stimulus intervals (ISIs) indicates the ability to generate calls with short inter-pulse intervals (IPIs).

Across *Xenopus* species, underlying laryngeal physiology is sometimes, but not always, consistent with behavioral sex differences. Sex differences in *Xenopus* laryngeal physiology have been characterized in three species, resident across the major clades of the *Xenopus* phylogeny. In *X. laevis*, male laryngeal muscle contracts more rapidly and displays higher potentiation values than female laryngeal muscle, which supports the production of rapid and intensity-modulated calls in males but not females (Tobias and Kelley, 1987). In *X. borealis*, differences in laryngeal physiology support reduced sex differences in vocalization: both male and female laryngeal muscle shows low EMG potentiation values and an inability to contract in response to short ISIs (Leininger et al., 2015). Unlike *X. laevis*, *X. borealis* calls are not rapid or intensity-modulated in either sex (Tobias et al., 2011, 2014). In a third species, *X. boumbaensis*, sex differences in laryngeal physiology are similar to that of *X. laevis.* However, male *X. boumbaensis* vocalizations more closely resemble those of *X. borealis* than *X. laevis* (Tobias et al., 2011) because EMG laryngeal potentiation mechanisms convert a burst-type brain pattern into single muscle contractions and sound pulses (Leininger and Kelley, 2013). The contrast between *X. borealis* and *X. boumbaensis* indicates that vocal features are not always accurate predictors of effector physiology (Leininger et al., 2015).

To date, sex differences in *Xenopus* laryngeal physiology have been characterized in species that have dimorphisms in IPI and IM that are either large or nonexistent. The question of how laryngeal physiology produces more subtle vocal sex differences is unclear. Here, we characterized vocalizations and laryngeal physiology of *X. muelleri*, a close relative of *X. borealis* (Evans et al., 2019). While both species have been shown to have reduced sex differences in call rapidity, *X. muelleri* males produce a more complex advertisement call (two sound pulses with ∼45 ms IPI) that, unlike the *X. borealis* advertisement call, is intensity modulated (Tobias et al., 2011, 2014). After recording vocalizations from several individuals, we used the isolated larynx preparation to compare laryngeal mass, EMG potentiation and PTT of the laryngeal muscle between males and females. Our predictions are informed by what is currently known about *X. muelleri* vocalizations, the species' location within the *Xenopus* phylogeny, and known sex differences in *Xenopus* laryngeal physiology. If sex differences in laryngeal physiology are evolutionarily conserved between *X. muelleri* and *X. borealis* regardless of behavior, we would expect relatively low levels of EMG potentiation and low PTT (at short ISIs) in both sexes. Less likely, laryngeal physiology may resemble sex differences seen in *X. laevis*, such as much greater EMG potentiation and PTT values in males relative to females. Finally, *X. muelleri* laryngeal physiology may not match either of these outcomes but may match observations from vocalizations. In this case, we would expect no sex difference in PTT (because of reduced sex differences in call rapidity), but greater EMG potentiation in males relative to females, owing to IM observed in male (but not female) calls.

MATERIALS AND METHODS

Organisms and housing

Xenopus muelleri (Peters 1844) originated from Xenopus Express (Brooksville, Florida). Frogs were housed at 22–24°C on a 12 h:12 h light:dark cycle in polycarbonate tanks (46×24×16 cm, lenght×width×depth) filled with 4% Holtfreter's solution (6.0 mmol l^{-1} NaCl, 67.1 µmol l^{-1} KCl, 83.1 µmol l^{-1} $MgSO_4$ $7H_2O$, 90.7 µmol l^{-1} $CaCl_2$ $2H_2O$, 0.23 mmol l^{-1} $NaHCO_3$) in unisex groups with no more than six frogs per tank. Frogs were fed twice a week with frog kibble, followed by a complete water change 3 h after feeding (Tobias and Kelley, 1987). The University of South Florida's Institutional Animal Care and Use Committee approved the housing, care and experimental protocols in this study (protocol R1500004543).

We recorded vocalizations during male–female pairings using a hydrophone (model H2A-XLR, Aquarian Audio Products) placed in the corner of testing tanks (60×32×30 cm) filled with 27–30 liters of 4% Holtfreter's solution (22–23°C). Males were injected with human chorionic gonadotropin (hCG) in two injections (100 IU hCG 18–24 h before recordings and 200 IU hCG 3 h before recordings) to their dorsal lymph sac to promote sexual readiness. Females were not injected. Vocalizations detected by the hydrophone were digitized by an Audiobox digitizer (sampling rate: 44.1 kHz; Audiobox USB 96, PreSonus) and recorded with Audacity (v. 2.3.0, audacityteam.org) on a laptop computer (HP ProBook 640 G3 Notebook PC). Vocalizations were recorded from three males for a period of 30 min.

Ex vivo larynx preparation

Nine male frogs (mass=13.59±0.76 g) and seven female frogs (mass=31.58±0.76 g) were given a terminal dose of 1.2% MS-222 (ethyl 3-aminobenzoate, methanesulfonic acid salt; 0.15 ml for males and 0.3 ml for females) by injection into the dorsal lymph sac. Anesthesia was confirmed by checking for a loss of righting reflex and movement. After anesthesia took effect, the frogs were placed in a bed of ice for 10 min and considered completely anesthetized when they no longer responded to a toe pinch. The larynx was removed from the ventral side of the body: after removing the layers of skin and muscle to expose the viscera, the anterior and posterior ends of the larynx were separated from the lower jaw and heart,

respectively, and removed from the body. Euthanasia was achieved during deep anesthesia by removal of the heart and immediate freezing of the rest of the body.

The isolated larynx was pinned dorsal side up in a shallow, Sylgard-lined Petri dish filled with a dextrose-supplemented Ringer's solution composed of 0.1 mol l^{-1} NaCl, 2 mmol l^{-1} KCl, 2.5 mmol l^{-1} $CaCl_2$ $2H_2O$, 3 mmol l^{-1} $MgCl_2$ $6H_2O$, 4 mmol l^{-1} HEPES and 27.7 mmol l^{-1} dextrose (Tobias and Kelley, 1987). Connective tissue, fat and blood vessels surrounding the larynx were removed using fine forceps and spring scissors to expose the laryngeal muscle, tendons and laryngeal nerve rootlets.

Electrophysiology setup

A handmade suction electrode was placed on the exposed laryngeal nerve rootlet, which delivered stimulation to the nerve based on input from an analog stimulus isolator (model 2200, A-M Systems). Placed between the laryngeal muscle and cartilage box, a bipolar silver wire electrode recorded EMG potentials, which were amplified by an extracellular AC/DC amplifier (gain=5 k, low pass filter=20 kHz, high pass filter=10 Hz; model 3000, AM-Systems). A force transducer (model FT03, Grass Technologies) was attached to the tendon connecting the laryngeal muscle to the cartilage discs, allowing us to record muscle tension in response to laryngeal nerve stimulation, via a bridge amplifier (model FE221, ADInstruments). All signals were digitized by a PowerLab 4/26 (model ML846, ADInstruments) and collected in the LabChart computer program. Within LabChart, separate channels recorded stimulation patterns, EMG potentials and tension transients with a 10 kHz sampling rate (Leininger et al., 2015).

We delivered a series of five doublet stimuli (0.1 ms stimulus pulse duration) to the laryngeal nerve to mimic the observed doublet advertisement call produced by intact male *X. muelleri*. The inter-doublet interval was 235 ms, based on the average inter-call interval for this species (Tobias et al., 2011). Each series was separated by a standard 2 min interval to allow for recovery of the larynx.

We altered the ISI between the two stimuli within a doublet in order to test whether ISI affects the strength of the EMG potentials and produces maintained or discrete tension transients. We tested a range of ISIs from 20 to 65 ms in male larynges and 20 to 80 ms in female larynges (which required longer ISIs to reach 100% tension), delivered in a randomized order. Following that experiment, we tested laryngeal responses to stimulus patterns that approximated both the observed pattern B call (see Fig. 1B) and female release calls (Tobias et al., 2011, 2014; Vigny, 1979): short trains of 15 stimulus pulses with various ISIs (48 ms for males; 50 ms for females). At the conclusion of each experiment, we trimmed excess cartilage from the larynx and measured its mass.

Data processing and statistical analysis

Unless otherwise noted, we analyzed data using the R statistical software (v. 4.0.0, R Core Team) together with the RStudio environment, and reported results as means±s.e.m.

Vocalizations

During vocal recordings, we observed two patterns (called 'A' and 'B'; Fig. 1A,B) that were noticeably different by temporal parameters; we analyzed them further to compare them quantitatively. We analyzed temporal qualities of vocalizations using LabChart (v. 8.0.10, ADInstruments) for all bouts of calling with a minimum of three calls, where the inter-call interval (ICI) did not exceed twice the average ICI of the preceding calls. We determined the call inter-pulse interval (IPI) by measuring the time difference between the maximum amplitude of each sound pulse (Fig. S1). We calculated the ICI by measuring the time difference between the maximum amplitude of the first sound pulses of adjacent calls. We calculated intensity modulation (IM) of doublet calls by dividing the difference in maximum amplitude of the second sound pulse and first sound pulse by the maximum amplitude of the first sound pulse (Fig. S1; Leininger et al., 2015; Tobias and Kelley, 1987). For calls with more than two sound pulses, IM was calculated using the highest amplitude sound pulse in lieu of the second sound pulse. The IPI, ICI and IM values of call patterns A and B were compared using a paired *t*-test to determine the extent to which they differ by temporal features. To characterize spectral features of calls, power spectra of pattern A and pattern B calls (n=3 individuals each) were created with Avisoft SASLab Pro (v. 5.2, Avisoft Bioacoustics) and plotted in R.

Laryngeal and body mass ratios

To determine the strength of the relationship between body and larynx mass, we computed the Pearson correlation coefficient for each sex. Larynx mass and larynx to body mass percentages were compared between sexes using a Mann–Whitney *U*-test.

Laryngeal muscle EMG

We computed a potentiation index (PI) of the EMG potentials to analyze the change in EMG response over a series of doublet stimuli. We calculated PI by dividing the difference in the maximum amplitude of the two peaks in a doublet by the maximum amplitude of the first peak (Fig. S1; Leininger et al., 2015). Across intervals, we observed a systematic difference in PI following the first stimulus burst relative to the others (bursts 2–5). Therefore, we averaged the PIs for bursts 2–5 and analyzed the first burst PIs separately from the averaged PIs from the rest of the doublets. Using SAS (v. 9.4), we modeled how the PI depends on sex, ISI and the burst number (first burst versus subsequent burst) using a linear mixed-effects model (SAS PROC MIXED). We assumed an autoregressive correlation structure of order 1 (AR1) for the serial observations on the same larynx. *F*-tests were used to assess the significance of the model parameters, including an ISI by sex interaction to determine whether the effect of ISI on the PI differs by sex. We also computed PI from the train stimulations delivered to five of the male larynges and seven of the female larynges. These values were compared using a non-parametric Mann–Whitney *U*-test.

Laryngeal muscle tension

For each ISI, we calculated the percent transient tension (PTT) to illustrate how readily the laryngeal muscle was able to produce discrete muscle contractions (where 100% indicates complete transient tension and 0% indicates completely fused tension). We calculated PTT for each ISI interval by dividing the difference between the first peak's maximum amplitude and the minimum amplitude of the trough between the first and second tension peak by the difference between the first tension peak's maximum amplitude and the baseline amplitude before the first tension peak (Fig. S1; Leininger et al., 2015). As before, we fitted a linear mixed-effects model to assess the effect of sex and ISI on the mean PTT, assuming an AR1 correlation structure to account for serial correlation between successive measures of PTT on the same larynx. An interaction term between sex and ISI was included in the model to determine whether the effect of ISI on the mean PTT differs by sex. Within a sex, we used Tukey *post hoc* tests to assess pairwise comparisons of mean PTT across ISIs.

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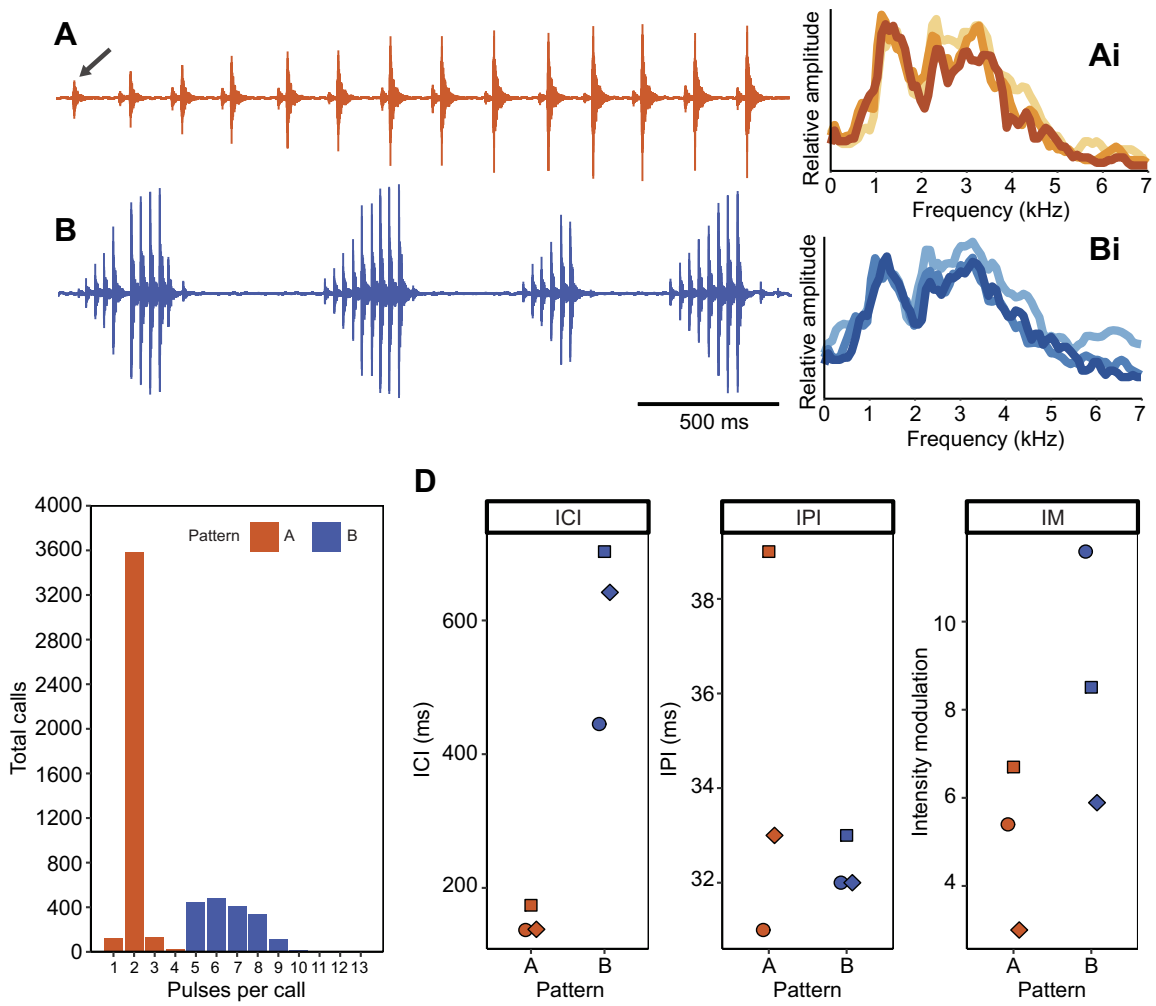

Fig. 1. Male *Xenopus muelleri* vocalizations when paired with an unreceptive female. (A) An oscillogram of vocal pattern A, the doublet advertisement call. Doublets are often preceded by a single sound pulse at the initiation of calling (arrow). Power spectra of a single call from three individuals are included on the right (Ai). (B) An oscillogram of vocal pattern B, a presumed approach call. Power spectra of a single call from three individuals are included on the right (Bi). Both oscillograms are from the same individual. Scale bar, 500 ms. (C) The distribution of number of pulses per call across total observations (n=3 frogs). Pattern A was mostly composed of two sound pulses per call and pattern B had between five and nine sound pulses per call (maximum=13 sound pulses). (D) Comparisons of inter-call interval (ICI), inter-pulse interval (IPI) and intensity modulation (IM) between vocal patterns A and B. Each frog is represented by a unique shape. The mean ICI of pattern B was significantly higher than that of pattern A (P=0.012). The mean IPI was not significantly different between patterns A and B (P=0.219). The mean IM was marginally significantly different between patterns A and B (P=0.055).

To estimate the threshold ISI value for which 50% PTT is achieved, we fitted a four-parameter log-logistic non-linear regression model for each sex. Such a model, popular in dose–response modeling, proposes a sigmoidal curve for the mean PTT. Using inverse regression, such models allow estimation of the ISI value, for each sex, for which the predicted PTT is 50% (or any other value). While the linear mixed-effect models mentioned above treat ISI as a discrete factor, the four-parameter log-logistic model treats ISI as a continuous predictor.

RESULTS

Identification of male *X. muelleri* vocalizations

We recorded vocalizations of three *X. muelleri* males (paired with unreceptive females) that were used in electrophysiology experiments. We successfully recorded at least 2 min of calling from each male per 30 min recording, which consisted of 10–30 bouts of calling for 3–56 s. We identified two distinct call patterns, designated as patterns A and B. The patterns have similar spectral profiles (Fig. 1Ai,Bi) but can be differentiated from one another by the number of sound pulses per call. The most common call pattern, pattern A, contained two sound pulses per call (Fig. 1A,C). The other call pattern, pattern B, had between five and nine sound pulses per call (Fig. 1B,C). Rarely (<2% of all calls), males produced single sound pulses, which occurred most frequently at the commencement of a bout of calling (81.4%), at the end of a bout of calling (7.1%), or as isolated pulses within a period of silence (11.5%) (Fig. 1A,C).

We compared the IPI, ICI, IM and spectral profiles of patterns A and B to determine the similarities and differences between the patterns, thus further characterizing features of *X. muelleri* male vocalizations. We compared the IPI, ICI and IM of patterns A and B to confirm that the patterns are different call types in the vocal repertoire of *X. muelleri* males. Although no significant difference was found between the IPI of patterns A (34.33±2.4 ms) and B (32.3±0.33 ms) (t=0.96, d.f.=2, P=0.219), there was a significant difference between the ICI of patterns A (149.6±12.2 ms) and B (596±77.9 ms) (t=6.4, d.f.=2, P=0.012) and a marginally significant difference between the IM of patterns A (5.03±1.08)

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and B (8.66±1.67) (t=2.75, d.f.=2, P=0.055) (Fig. 1D). When comparing the spectral profiles of patterns A and B with previously published vocalizations elicited from male–female pairings, pattern A resembles the *X. muelleri* male advertisement call (Tobias et al., 2011) and pattern B, first described in Vigny (1979), is most likely a *X. muelleri* male approach call. Furthermore, the spectral profiles of these patterns are distinct from those characteristic of female release calls (Tobias et al., 2014).

Anatomy of the *X. muelleri* larynx is sexually dimorphic

The structural component of the larynx is a rigid, air-filled cartilage box. The box is divided into five chambers and the chamber posterior to the glottis contains a pair of sound-producing arytenoid cartilage discs. A pair of bipennate muscles flank the outside of the laryngeal box and insert onto a ridge of the cartilage discs via the laryngeal tendon (Fig. 2A). All vocalizations are produced without respiration when the tendon of the bipennate muscle pulls on and separates the cartilage discs (Kwong-Brown et al., 2019).

The male bipennate laryngeal muscles and cartilage are markedly larger than the female muscles (Fig. 2A). *Xenopus muelleri* male larynges (258±13 mg) weighed approximately 1.74 times more, on average, than female larynges (148±8.8 mg; Mann–Whitney U-test, P<0.001). The larynx accounted for an average 1.9% of the total body mass of males, a 4-fold increase compared with the female's 0.47% (Mann–Whitney U-test, P<0.001; Fig. 2B). In males, body mass and larynx mass were found to have a significant positive correlation, with males with larger body masses possessing heavier larynges (R=0.88, P=0.002). In females, body mass and larynx mass were not significantly correlated (R=0.314, P=0.490; Fig. 2C).

Laryngeal response to call-like stimulation

Using an *ex vivo* larynx preparation (Tobias and Kelley, 1987; Leininger et al., 2015), we characterized the laryngeal muscle responses to ethologically relevant stimulus patterns. A series of doublets with ISIs equal to previous descriptions of male IPIs (45 ms) produced discrete tension peaks (Fig. 3A; Tobias et al., 2011). With the exception of the first doublet, male laryngeal muscle completely contracted and relaxed in response to each stimulus pulse per doublet, a response that would generate two sound pulses. When we delivered the same stimulation pattern to isolated female larynges, doublets at ISIs of 50 ms produced a series of discrete tension peaks (Fig. 3B). Therefore, for both sexes, the *X. muelleri* larynx is capable of converting doublet neural stimulations into two discrete tension transients that would lead to the production of a burst advertisement call (even though intact *X. muelleri* females do not produce such vocalizations).

We also delivered a train of stimuli that mimicked the pattern B calls observed during behavioral recordings. From a train of 15 stimuli, male larynges (n=5) generated 14 discrete tension transients (Fig. 3C). Therefore, male larynges are capable of converting a neural stimulus train at an ISI equivalent to the IPI of the observed pattern B call into discrete tension transients, except for a failure to convert the first stimulus. We also delivered trains mirroring previously published parameters of the female release call

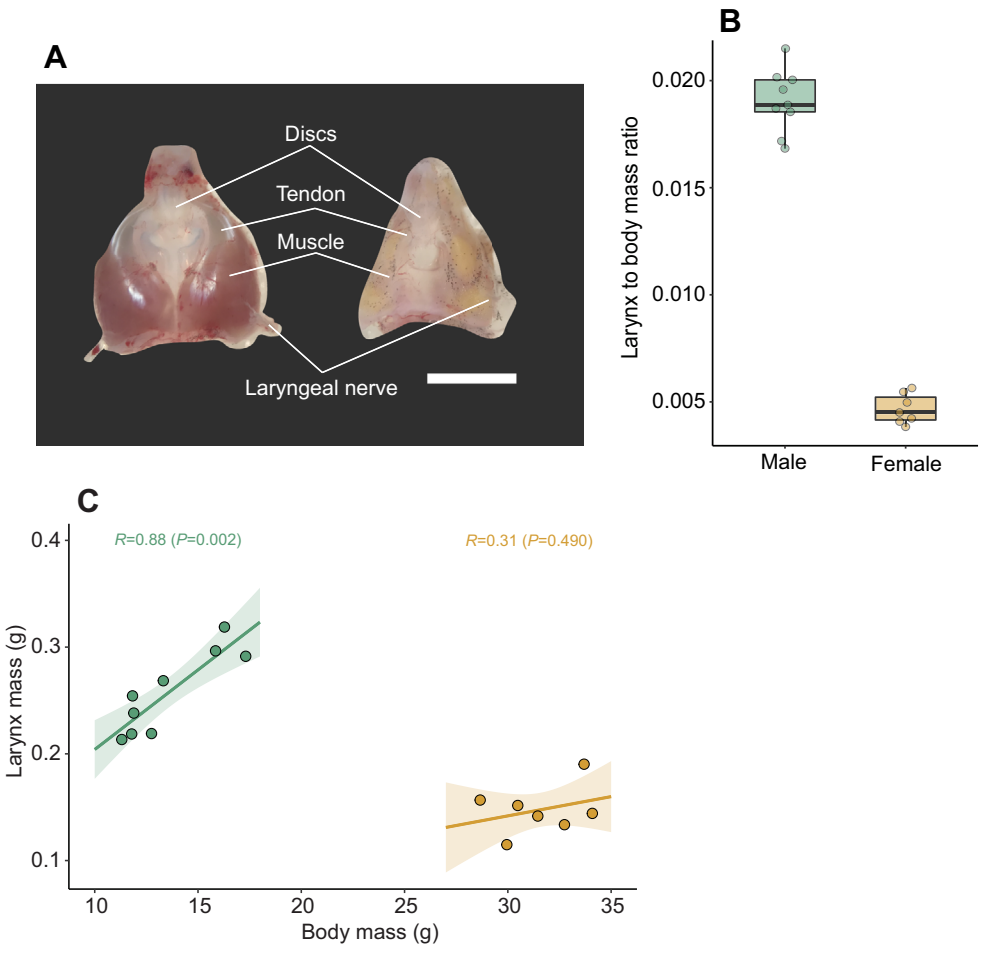

Fig. 2. Sex differences in *X. muelleri* laryngeal size and body mass scaling. (A) Dorsal view of the *X. muelleri* male (left) and female (right) larynx. The larynx is bilaterally symmetrical. A tendon connects the bipennate laryngeal muscles to the sound-producing cartilage discs contained within the cartilage box. The laryngeal nerve innervates the laryngeal muscle at the posterior end of the larynx. Scale bar, 5 mm. (B) The male larynx to body mass ratio is significantly greater than the female larynx to body mass ratio (n=7 for females and n=9 for males; P<0.001). The dots represent individual observations and the box plot represents the distribution. (C) There is a significant positive correlation between laryngeal mass and body mass in *X. muelleri* males (R=0.88, n=9, P=0.002) but not in females (R=0.31, n=7, P=0.490).

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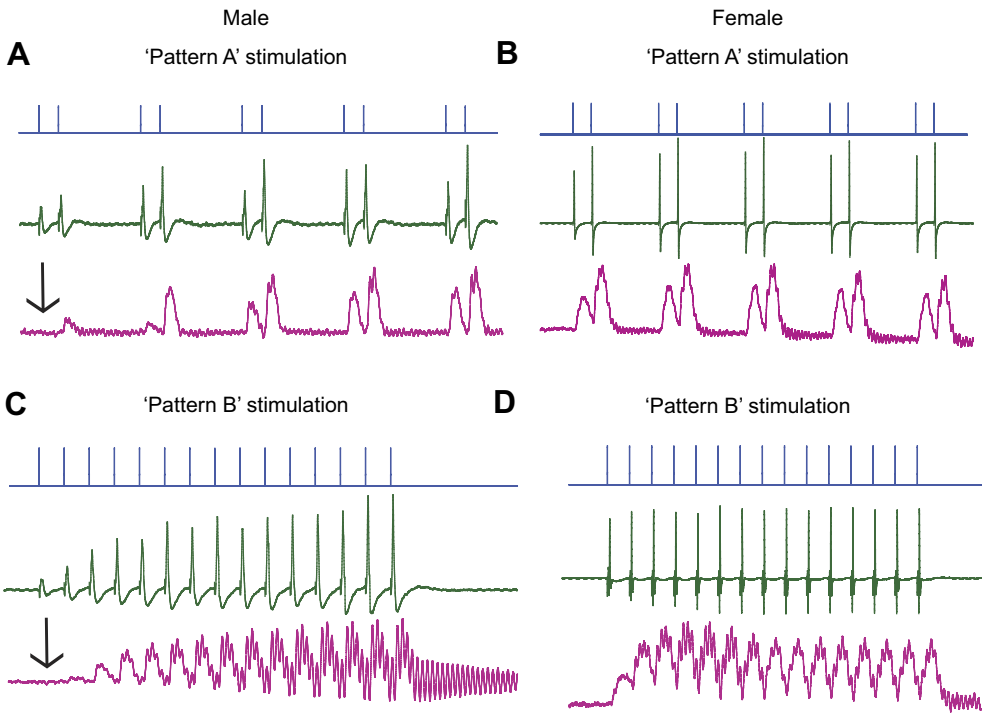

Fig. 3. Laryngeal responses to ethologically relevant stimulations in *X. muelleri* males (left) and females (right). (A) In response to a stimulus mimicking the advertisement call [pattern A; 2 pulses, 45 ms inter-stimulus interval (ISI)], the male larynx is capable of producing robust electromyogram (EMG) potentials and discrete tension peaks, except for the first stimulus of the first burst (arrow). (B) Although female *X. muelleri* do not produce burst advertisement calls, their larynx is capable of producing discrete tension peaks at stimulation intervals equivalent to their release call IPI (50 ms). (C) Similar to A, in response to a stimulus mimicking the approach call (pattern B, 15 pulses, 45 ms ISI), the male larynx produced very small EMG and no muscle tension at the start of stimulation. (D) In response to a stimulus mimicking the female release call (train of 50 ms ISI; Tobias et al., 2014), the female larynx was capable of producing EMG potentials and discrete tension peaks. The high-frequency component of the tension records reflect intrinsic vibration of the string within the force transducer. Trace order: top (blue), stimulus; middle (green), EMG; bottom (magenta), tension.

(IPI=∼50 ms; Tobias et al., 2014) to isolated female larynges (n=7). Female larynges produced discrete tension transients to all stimulus events within the train (Fig. 3D). Therefore, female larynges are capable of converting neural stimuli resembling the female release call into discrete tension necessary for vocal production.

In male larynges, we observed that the muscle's response to the first stimulus reliably differed from the response to subsequent stimuli. Regardless of the ISI, the first doublet stimulus of pattern A trials produced a single small tension transient at the end of the stimulus doublet. Additionally, the first stimulus of the pattern B train did not produce a tension peak, whereas subsequent stimuli did. The observation of single sound pulses consistently produced at the start of a bout of pattern A calling potentially mirrors the production of a single tension peak in response to the first doublet stimulation.

Sex differences in EMG potentiation

We assessed sex differences in *X. muelleri* laryngeal EMG potentiation over a range of ISIs both above and below ethologically relevant values. Across individuals, PI values were systematically higher in response to the first stimulus doublet within a series (owing to the small amplitude of the first EMG) whereas subsequent doublet stimuli had lower PI values. To simplify our analysis, we used the PI from the first burst but averaged the PI from the four subsequent bursts, so the dots in the second panel of Fig. 4A represent the average PI values over the four subsequent bursts.

Our linear mixed-effects model, as illustrated in Fig. 4A, revealed no interaction between sex and ISI ($F_{9,141}$=1.33, P=0.226) and no effect of ISI ($F_{12,141}$=0.69, P=0.790) on PI. We did find a statistically significant effect of sex ($F_{1,14}$=12.0, P=0.004), although the effect was very small: male PI values were higher than female PI values by less than 1.0, across ISIs for both first and subsequent bursts. We found a significant difference in the mean PI between the first and subsequent burst ($F_{1,15}$=18.4, P<0.001), with the mean PI of the first burst estimated to be 0.61 higher than the mean PI of the subsequent bursts.

We also delivered train stimulation patterns that modeled the observed pattern B call to male larynges (ISI=48 ms; n=5) and the female release call to female larynges (ISI=50 ms; n=7). Male laryngeal PI was highly variable between individuals while female PIs were fairly consistent (Fig. 4B). In response to these train stimuli, the median male PI (4.6) was significantly higher than median female PI (1.2) (Mann–Whitney U-test; P=0.003).

Sex differences in laryngeal muscle tension

We made tension recordings from the tendon connecting the laryngeal muscle to the sound-producing cartilage discs to determine the range of ISIs over which male and female laryngeal muscle can produce discrete tension transients (Tobias and Kelley, 1987). Unlike the trend observed in the EMG PI values, PTT values for each ISI remained constant for each stimulus throughout the series. Therefore, we calculated a mean PTT value at each ISI for each larynx. For all larynges, we observed instances of 100% transient tension for ISIs at and above 50 ms, with lower ISIs resulting in varying degrees of maintained tension (Fig. 5). At the three lowest intervals, the majority of observed larynges displayed less than 50% PTT. The highest variability in the PTT occurred in

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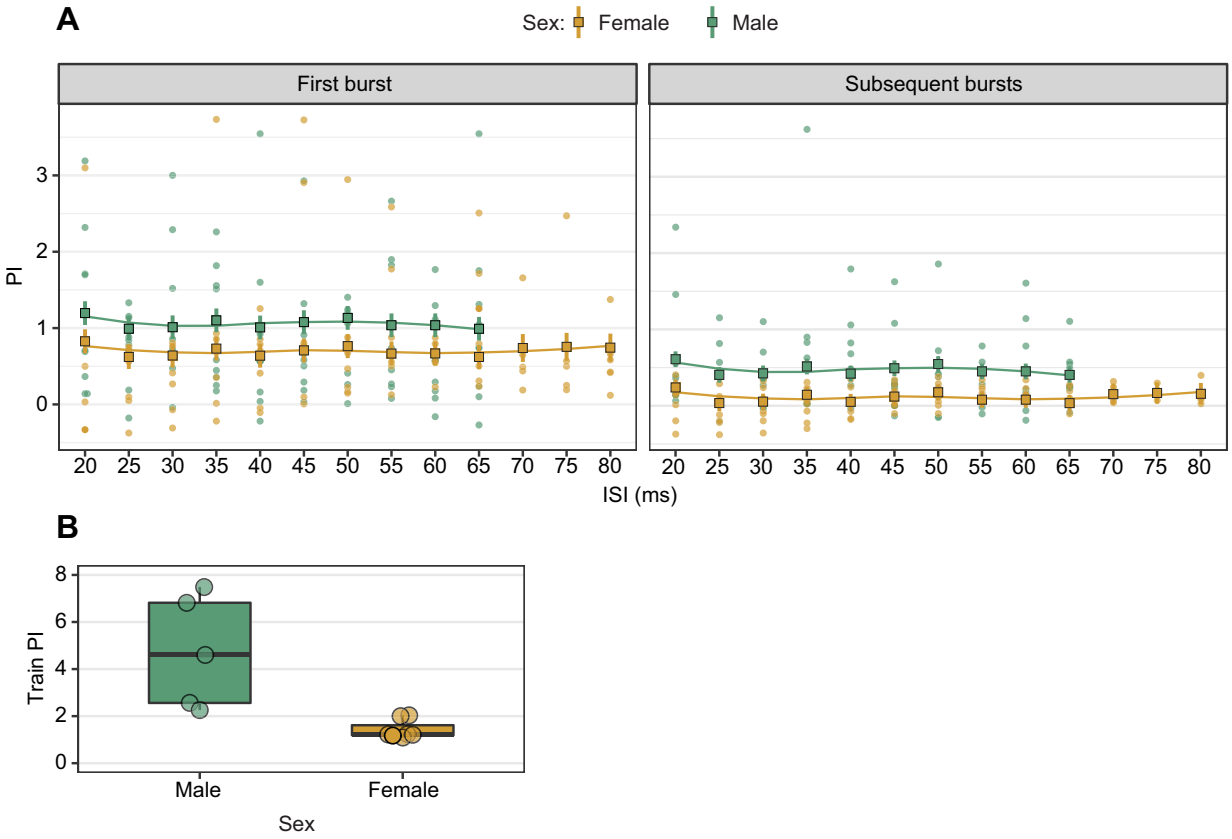

Fig. 4. EMG potentiation in *X. muelleri* male and female larynges. (A) Observed and fitted potentiation index (PI) for male and female EMGs in response to the doublet stimulation pattern over a range of ISIs. The faint dots represent the PI measured from each individual (male n=9, female n=7). Squares represent the fitted PI for each sex obtained from a linear mixed-effects model, with the error bars indicating ±1 s.e.m. There was no effect of ISI on the fitted PI (P=0.790), and a small but statistically significant effect of sex (P=0.004). In both sexes, the fitted PI in response to subsequent bursts is smaller than the fitted PI in response to the first burst (P<0.001). (B) In response to a longer stimulus train (15 pulses at 48–50 ms ISI), the median PI for males (n=5, median=4.6) is significantly higher than the median PI for females (n=7; median=1.2, P=0.003). The dots represent the individual observations and the box plot represents the distribution (median, quartiles, range).

response to 35–45 ms in males and 40–50 ms in females; these ISIs are within the range of IPIs of our call recordings and published values (Fig. 1; Tobias et al., 2011; Tobias et al., 2014).

Our linear mixed-effects model revealed a significant interaction between ISI and sex ($F_{9,141}$=2.36, P=0.016), with some of the largest estimated differences in PTT between males and females occurring at ISIs of 35 ms (difference of 26%), 40 ms (23%) and 45 ms (24%). Differences for ISI levels below 35 ms were 20% or less. Differences in PTT for ISI levels larger than 45 ms were 15% or less (Fig. 6).

When fitting models to each sex, Tukey *post hoc* tests revealed that the mean PTT for the two lowest intervals (20 and 25 ms) were not significantly different from each other. Similarly, the mean PTT did not differ significantly between the highest intervals (50–65 ms for males and 60–80 ms for females). For both sexes, the 35 ms interval's PTT values were shown to be significantly less than those of all higher ISIs (P<0.001).

Using the fitted four-parameter log-logistic model (Fig. 6), we were able to estimate the horizontal shift between the sexes for a given PTT. The estimated threshold ISI necessary to elicit 50% PTT equals 30.1±2.5 ms in males and 40.8±1.8 ms in females. For 80% PTT, the thresholds are estimated as 43.2±1.8 ms for males and 54.8±1.7 ms for females. This suggests a difference of approximately 11 and 12 ms in the ISI that is necessary to produce 50% and 80% PTT, respectively, between males and females (Fig. 6).

DISCUSSION

In *Xenopus*, examining how laryngeal physiology generates behavior has previously revealed either extreme sex differences in physiology in most species examined or a lack of sex differences in certain physiological mechanisms in one species (*X. borealis*). In this study, we have identified laryngeal sex differences in *X. muelleri*, a close evolutionary neighbor of *X. borealis*, that are unique from what has been described in other *Xenopus* species. We will discuss sex differences present in the laryngeal morphology, PTT, EMG potentiation and vocalizations of *X. muelleri*, and propose physiological and developmental mechanisms that may underpin these differences within an evolutionary framework.

Laryngeal mass: sex and species differences

Across *Xenopus* species that have been studied, male larynges are larger than female larynges by both gross mass and percentage of body mass, though the magnitude of these differences is variable. In our study, the *X. muelleri* larynx accounted for 1.9% of male body mass and 0.47% of female body mass (~4-fold difference). This is most similar to differences in *X. laevis*, where the larynx is 1.6% of male body mass and 0.3% of female body mass (~5-fold difference; calculated from Tobias et al., 1991). These ratios are less than that of *X. borealis* (2.47% for males and 0.245% for females; ~10-fold difference) and *X. boumbaensis* (8.18% for males and 0.42% for females; ~19.5-fold difference; Leininger et al., 2015). The phylogenetic trends and ontogenetic reasons underlying variation

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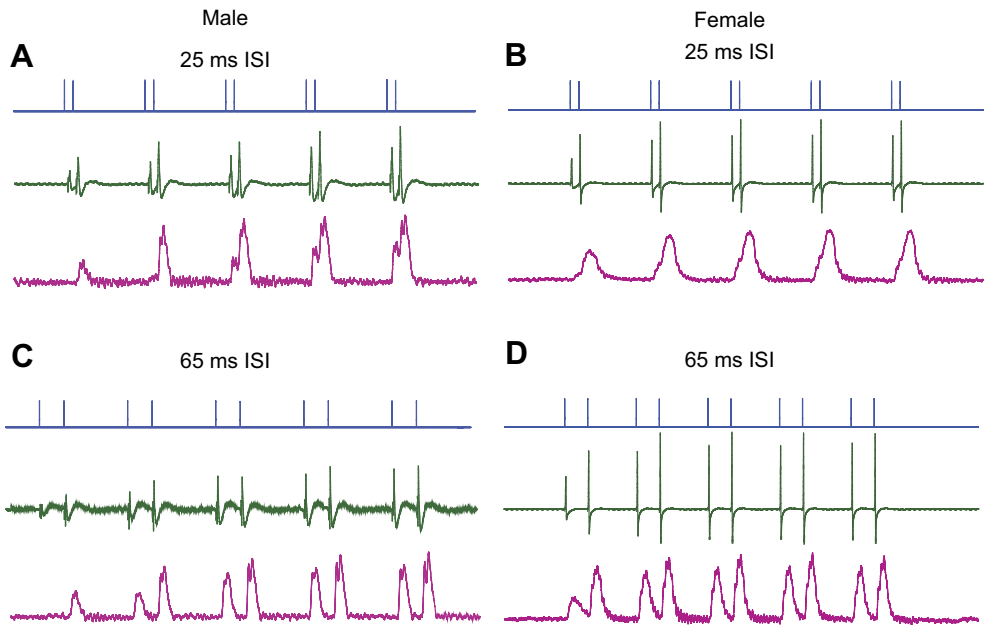

Fig. 5. Maintained and discrete laryngeal muscle tension in response to small and large ISIs in male (left) and female (right) larynges. In response to doublet stimuli of 25 ms ISI (top row), male (A) and female (B) larynges are not capable of producing fully discrete tension transients, but exhibit slight differences in percent transient tension (quantified in Fig. 6). In response to doublet stimuli of 65 ms ISI (bottom row), male (C) and female (D) laryngeal muscle is capable of producing discrete tension transients, indicating an ability to fully contract and relax. Trace order: top (blue), stimulus; middle (green), EMG; bottom (magenta), tension.

in laryngeal sex differences are not yet known. For example, in two species of hylid, species differences in laryngeal sexual dimorphism are driven mainly by variation in male laryngeal mass rather than female laryngeal mass (McClelland et al., 1997). Based on the present data, it is plausible that variation in *Xenopus* laryngeal sex differences are also driven by male laryngeal mass, but a more robust sampling of species is needed to reveal phylogenetic trends. Future studies could also determine whether diversity in laryngeal sex differences correlates with any aspects of the vocal repertoire.

Laryngeal transient tension: behavioral and evolutionary implications

Xenopus laryngeal muscle must complete cycles of contraction and relaxation for production of multiple sound pulses. The ability of the muscle to contract and relax with high PTT (also known as percent relaxation) in response to short ISIs allows for production of rapid calls with short IPIs, a feature characteristic of males in most *Xenopus* species. In most species of *Xenopus* studied, PTT is a sexually dimorphic feature of laryngeal muscle, with the exception of *X. borealis*, where PTT is monomorphic (Leininger et al., 2015; Tobias and Kelley, 1987). In *X. muelleri*, we found that sex differences in PTT are unique compared with other species studied to date (Fig. 7). Both sexes exhibited a sigmoidal relationship between ISI and laryngeal PTT, such that larynges of both sexes could not produce discrete tension at short ISIs. However, the relationship was shifted slightly towards shorter intervals in males relative to females, suggesting that male larynges may be able to produce vocalizations that are slightly – but not extremely – more rapid than female vocalizations.

Laryngeal muscle transient tension is based in part on the complement of myosin heavy-chain genes that are expressed, which may differ between sexes and across species. In *X. laevis* and *S. tropicalis*, expression of an androgen-sensitive myosin heavy-chain gene results in exclusively fast-twitch muscle fibers in the

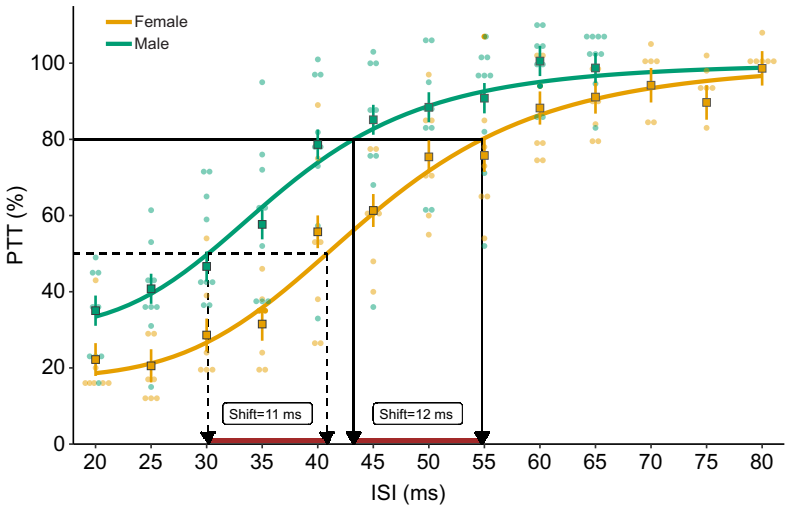

Fig. 6. Observed and fitted percent transient tension (PTT) of *X. muelleri* male (*n*=9) and female (*n*=7) laryngeal muscle at various levels of ISI. PTT was at or close to 100% at longer (60 ms or higher) ISIs. In both sexes, PTT decreased as ISI shortened, with the largest changes in PTT occurring between 35 and 40 ms ISI. There was a small but significant interaction between sex and ISI on PTT (P=0.016), and male PTT values were higher than female PTT values at most ISIs. The faint dots represent the observed PTT for each individual. The squares represent the fitted PTT for each sex obtained from a linear mixed-effect model, with the error bars indicating ±1 s.e.m. The smooth curves represent the fit of the four-parameter log-logistic model and illustrate the shift in the ISI threshold between males and females that is necessary to produce a given PTT (a shift of 11 ms for 50% PTT and 12 ms for 80% PTT).

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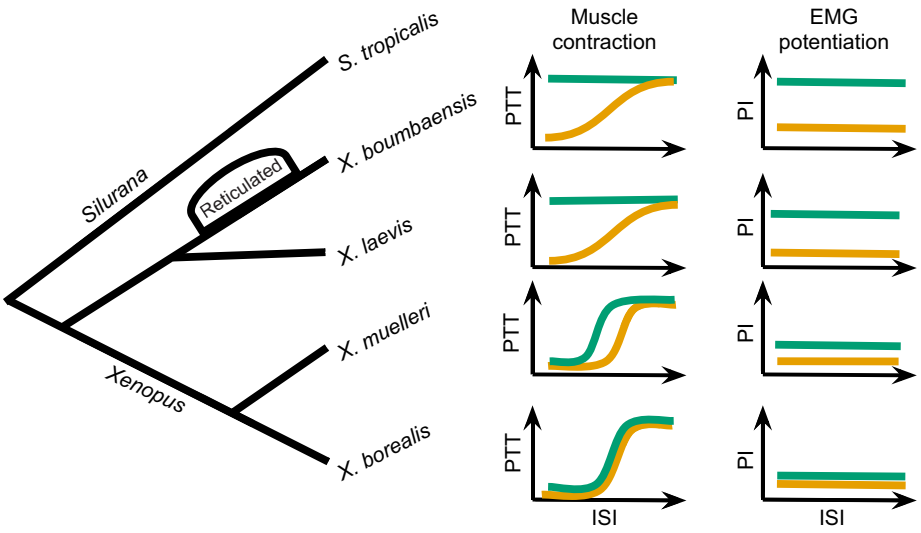

Fig. 7. Summary of known sex differences in *Xenopus* laryngeal physiology within a phylogenetic context. The genus *Xenopus* includes three major species groups: a reticulated clade that includes *X. boumbaensis*, a clade that includes *X. laevis*, and a clade that includes *X. borealis* and *X. muelleri*. The three clades share one most recent common ancestor (MRCA) dating to 45 mya; the MRCA of *X. borealis* and *X. muelleri* dates to 20 mya and the MRCA of *X. boumbaensis* and *X. laevis* dates to 25 mya. Both *X. laevis* and *X. boumbaensis* display high sexual dimorphism in laryngeal muscle contraction and EMG potentiation. *Xenopus borealis* displays sexually monomorphic laryngeal muscle contractions and EMG potentiation. In contrast, *X. muelleri* displays an intermediate phenotype in that the muscle contractions and EMG potentiation show a smaller degree of sex difference (although the overall trends are similar to its close relative *X. borealis*). Phylogeny and MRCA dates are adapted from Evans et al. (2019). Schematic responses: teal, male; orange, female.

male larynx, allowing for rapid contraction and relaxation and production of rapid calls (Baur et al., 2008; Catz et al., 1992). Further molecular characterization of *X. muelleri* myosin heavy-chain gene sequence and expression patterns will help explain the subtle sex differences in PTT that we observed.

Laryngeal EMG potentiation: behavioral and evolutionary implications

Laryngeal EMG potentiation is a peripheral mechanism that contributes to the production of intensity-modulated *Xenopus* calls, a sexually dimorphic vocal feature across many *Xenopus* species. In *X. muelleri*, we found that sex differences in laryngeal EMG potentiation is distinct from that found in other species studied to date. Laryngeal EMG potentiation in male larynges was significantly greater than that of female larynges, but the magnitude of the difference was small relative to other species. Overall EMG potentiation values were slightly higher than those described in *X. borealis* (Leininger et al., 2015) and stand in stark contrast to the marked sex differences in EMG potentiation observed in *X. boumbaensis* (Leininger et al., 2015) and *X. laevis* (Tobias and Kelley, 1987). Both vocal patterns that we recorded from *X. muelleri* males contained some degree of IM, and the patterns differed significantly in the degree of IM. Alongside motor neuron recruitment in the vocal pattern generator, EMG potentiation in the laryngeal muscle can help generate these intensity-modulated calls (Yamaguchi and Kelley, 2000).

In the male *ex vivo* larynx, EMG potentiation in response to the first stimulus burst was significantly greater than the response to subsequent bursts. In addition, the laryngeal muscle often failed to produce a contraction in response to the first stimulus, but reliably produced muscle contractions in response to subsequent stimuli. While we cannot rule out an artefact of the *ex vivo* preparation, the results may explain an interesting behavioral observation from calling males. Males occasionally produced single sound pulses, mostly at the start of a bout of calling or as stray isolated pulses. Synthesizing these observations, the unique EMG response at the start of a stimulus burst may also occur *in vivo*, resulting in a single muscle contraction and sound pulse. Observations of laryngeal EMG potentiation in *X. laevis* and *X. boumbaensis* are consistent with this possibility. In *X. laevis*, the larynx produces contractions and sound only after a few initial stimuli from the nerve (Tobias and Kelley, 1987; Yamaguchi and Kelley, 2000), after which EMG potentiation is sufficient to sustain muscle contractions and pulse production. In *X. boumbaensis*, the brain produces doublet stimuli, which the larynx converts into single muscle contractions and single sound pulses; the interval between doublets is sufficiently long (1 s) to exceed the time frame for potentiation across calls (Leininger and Kelley, 2013).

Evolution of sexually dimorphic physiology and hormonal regulation

How do behavioral and physiological sex differences evolve over time? Sex differences in behavior may be due to physiological differences in the nervous system, in target organs, or in both of these. Steroid hormones are a global signal that can coordinate the sexual differentiation of neuromuscular circuits, and changes in the amount, localization and targets of these signals may give rise to differences in sexually differentiated behaviors. Fuxjager et al. (2018) present several non-mutually exclusive hypotheses including evolutionary changes to circulating hormone levels, either globally or tissue-specific, changes to hormone receptor distribution across tissues, or changes to the downstream gene network targets of hormone receptors. This framework allows us to generate hypotheses about the evolution of sexually dimorphic laryngeal physiology and behavior in *Xenopus* based on the present data.

In *X. laevis* and *S. tropicalis*, androgens shape many sexually differentiated features of the larynx from a female-like developmental default, resulting in male larynges possessing a

larger overall mass, larger muscle fiber size and number, a larger proportion of fast-twitch muscle fibers, and greater complexity and amount of cartilage. Together, these androgen-regulated features support prolonged and rapid male vocalizations with spectral signatures (Baur et al., 2008; Marin et al., 1990; Sassoon and Kelley, 1986). In each *Xenopus* species examined to date, male larynges are significantly larger than female larynges in terms of gross mass and as a percentage of body mass, indicating that laryngeal androgen responsiveness is conserved to at least some degree. That said, subtleties in the range of sex differences in laryngeal mass that we have discussed previously could reflect evolution of hormonal and developmental factors. For example, circulating androgen levels and/or the laryngeal tissue's degree of responsiveness to androgens may affect the degree of sex differences in laryngeal mass.

Developmental factors may be altered across species to result in various degrees of sex differences. In *X. laevis* and *S. tropicalis*, androgens developmentally regulate sex differences in laryngeal muscle coupled with fiber type switching via a population of androgen-sensitive satellite cells (Nasipak and Kelley, 2012). Species such as *X. muelleri* and *X. borealis* exhibit sex differences in overall laryngeal mass but reduced differences in muscle contractile abilities. Therefore, it is possible that laryngeal muscle satellite cells still function to support muscle fiber proliferation (leading to sex differences in muscle mass and muscle fiber number) but play a reduced role in fiber type switching in species such as *X. borealis* and *X. muelleri*. Alternatively, the role of satellite cells in fiber type switching could remain intact, but the downstream gene targets (such as laryngeal myosins) could be less dimorphic than what is seen in other species.

Some behavioral features, such as call IM, can be regulated by multiple hormone-mediated mechanisms. For example, estrogens regulate laryngeal synaptic strength from a male developmental default to support a stronger neuromuscular junction in females relative to males in *X. laevis* (Tobias and Kelley, 1995). This sexually differentiated feature supports production of intensity-modulated male vocalizations (via facilitation at the neuromuscular synapse). However, *X. laevis* intensity-modulated calls are also generated in part by motor neuron recruitment (Yamaguchi and Kelley, 2000), which is androgen-regulated (Rhodes et al., 2007). Species differences in estrogen- versus androgen-regulated mechanisms of IM could explain species-specific variation in call IM (Tobias et al., 2011).

In all *Xenopus* species studied to date, steroid hormones regulate sex differences in body mass and laryngeal mass (Zornik and Yamaguchi, 2011). Each species may have various degrees of hormonal control of physiological mechanisms in order to generate diverse behaviors. Unlike most species of *Xenopus* studied to date, *X. borealis* lacks the marked sex differences in laryngeal EMG potentiation (a proxy for synaptic strength) and laryngeal muscle fiber type and transient tension observed in species such as *X. laevis* and *X. boumbaensis* (Leininger et al., 2015; Fig. 7). The reduced sex differences in laryngeal physiology in *X. borealis* suggest a selective loss of hormonal regulation of these features while leaving hormonal regulation of other features (such as overall larynx size) intact. *Xenopus muelleri* presents an intermediate phenotype, in that sex differences in EMG potentiation and laryngeal muscle tension are present but subtle. The reasons underlying this phenotype are unclear, but could be due to reduced circulating hormones or hormone sensitivity, as well as changes in hormonally regulated gene expression (Fuxjager et al., 2018). For example, golden-collared manakins, which have an elaborate androgen-dependent 'wing-snap' display, have elevated expression of androgen receptors in their skeletal muscle and also more androgen-responsive genes relative to the skeletal muscle of zebra finches, which do not perform this display. Furthermore, androgen-induced differential gene expression also varied across the muscles involved in the wing-snapping display, indicating that the target effects of androgen can be tuned towards each muscle's function (Fuxjager et al., 2016). In *Xenopus*, future comparative studies of circulating steroid hormone levels, hormone receptor expression and transcriptomic targets can help us understand how hormonal regulation of muscle physiology has evolved to support diverse behavioral repertoires. Because androgens and estrogens both help direct sexual differentiation of laryngeal physiology, it may be that these pathways are regulated uniquely across species, resulting in the spectrum of sex differences of various laryngeal features.

The historical prioritization of describing and studying highly sexually dimorphic systems may mislead us to think that 'with respect to any trait, the sexes are either fundamentally different or they are the same' (Maney, 2016). Our study describes a species of *Xenopus* in which smaller effects of sex on underlying laryngeal physiology can shape vocalizations in a subtle manner. Further characterization of additional *Xenopus* species are necessary to determine how widespread this phenotype may be across the genus, and how physiological mechanisms that regulate sex-specific vocalizations have evolved.

Acknowledgements
We thank Quinn Ferguson, Susie Hajmohammad and Robyn McCartan for assistance with animal care. We also thank Ben J. Evans for advice on phylogenies, and Erik Zornik and three anonymous reviewers for helpful comments on an earlier version of the manuscript.

Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: K.E.S., E.C.L.; Methodology: K.E.S., E.C.L.; Software: K.E.S., B.K.; Validation: K.E.S., B.K., E.C.L.; Formal analysis: K.E.S., B.K.; Investigation: K.E.S.; Resources: E.C.L.; Data curation: K.E.S.; Writing - original draft: K.E.S.; Writing - review & editing: K.E.S., E.C.L., B.K.; Visualization: K.E.S., B.K., E.C.L.; Supervision: E.C.L.; Project administration: E.C.L.; Funding acquisition: E.C.L.

Funding
We acknowledge internal start-up funding from New College of Florida and the New College Women's Giving Circle (E.C.L.) that made this study possible.

Data availability
Data for this study are available from the Dryad digital repository (South et al., 2021): dryad.tdz08kpzr.

Supplementary information
Supplementary information available online at
https://jeb.biologists.org/lookup/doi/10.1242/jeb.231712.supplemental

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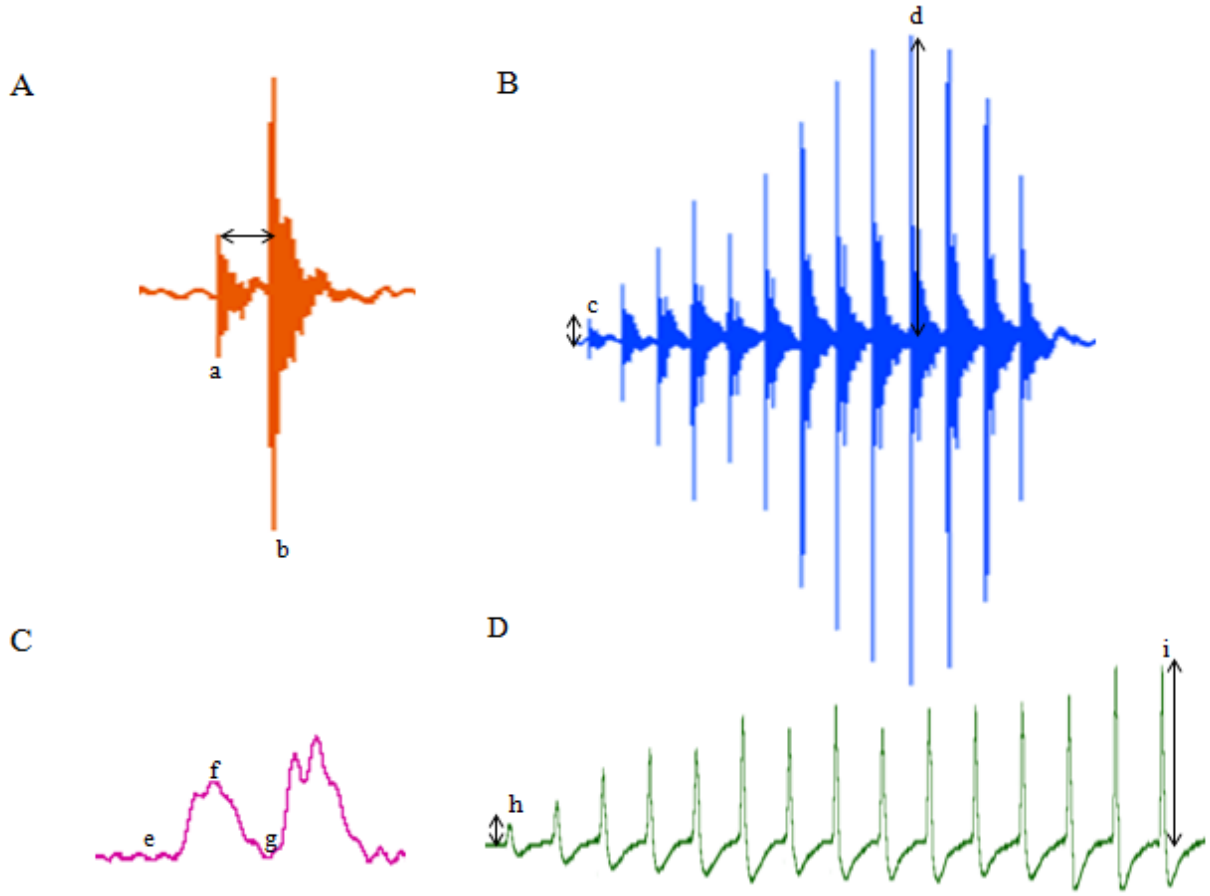

Figure S1. Quantifications of electrophysiology and vocal recordings. A) The inter-pulse interval (IPI) of vocal recordings are obtained by finding the time difference between the maximum amplitudes of the sound pulses of each call (*b-a*). For pattern B trains, the average interval between all pulses is the IPI of the call. **B)** The intensity modulation (IM) of vocal recordings are calculated by finding the difference in maximum amplitude between the largest sound pulse and the first sound pulse and dividing the difference by the amplitude of the first sound pulse (*(d-c)/c*). **C)** The percent transient tension (PTT) of the laryngeal tendon recordings is calculated by dividing the difference in amplitude between the first tension peak and the trough between the first and second tension peaks by the difference in amplitude between the first tension peak and the baseline tension (*(f-g)/(f-e)*). **D)** The potentiation index (PI) of laryngeal electromyograms (EMGs) is obtained by dividing the difference in maximum amplitudes of the tallest EMG and first EMG by the maximum amplitude of the first EMG (*(i-h)/h*).