

Ethology and evolution of courtship vocalization in *Xenopus*

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Abstract

Effective communication requires coordinated exchanges of socially appropriate signals. Yet, little is understood of how these exchanges have evolved. *Xenopus laevis* engage in vocal duets during courtship, which is uncommon in anurans. Males produce advertisement calls composed of two trills differing in rate and duration to attract female. Sexually-receptive females respond with rapping, a series of clicks. Males answer by shortening the slower trill whose rate can overlap that of rapping, and intensifying and extending the faster trill. While males across the *Xenopus* genus produce species-specific advertisement calls, not all are biphasic. Apart from *X. laevis* South Africa, it is unknown whether other species in the *Xenopus* genus perform duets. Herein, we investigate the occurrence and structure of courtship duets across four species in the L clade (*X. laevis* South Africa and Malawi, *X. petersii*, *X. victorinus*, *X. poweri*) of the *Xenopus* genus. Through acoustic recordings, we quantitatively characterized male-female vocalizations during courtship. We observed that males differentially modify their advertisement calls in response to conspecific rapping. For monophasic callers, calls are faster and intensified. For biphasic callers, the faster trills are intensified and either extended or more frequent. Interestingly, we observe that female conspecifics of biphasic callers preferentially rap during one of the two calls. Results support a model in which sexual selection may have acted through vocal exchanges to promote speciation in *Xenopus*.



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1. Introduction

The hallmark of social interactions is the ability of turn-taking in animals. Individuals involved in the communication coordinate the timing of acoustic exchanges based on the others' behaviors and social contexts (1). While being a fundamental organizing component of human speech, the trait of turn-taking can be found in organisms across taxa, including insects, amphibians, birds, and mammals (2, 3). Though turn-taking has prominent effects in social functions that directly influence species evolution (including competition and courtship) (4), there currently exists little research on the role of turn-taking in evolution.

Xenopus, or African clawed frog, engages in turn-taking behaviors through male-female courtship duets. Native to sub-Saharan Africa, *Xenopus* mainly lives in ponds with low visibility and high population density (5). Vocal communication therefore dominates *Xenopus* social interaction. The genus includes 29 species divided into two sub-generes: *Silurana* (including 4 species) and *Xenopus* (including 25 species from three clades: A, L, and M) (6), and the vocal signal produced by each species is highly species-specific. With many of these species available for experimental study, this frog genus provides a highly tractable system for examining the relationship between vocal turn-taking and evolution.

Each species of male *Xenopus* produces a distinct advertisement call for attracting females, and these calls differ in two ways: spectral features (pitch) and temporal patterns (rhythms) (7). For spectral features, each species has two distinct dominant frequencies, while the ratio between the two frequencies is shared within the clade (8). For temporal patterns, each species call is uniquely identified by combinations of the four general patterns: click, burst, trill, and biphasic. These patterns are distinguished by differences in call rate, click number, and intensity modulation (9). More specifically, trills consist of fast trill and slow trill, which are sound pulses repeated at 60 Hz and 30 Hz respectively (10). The advertisement calls are critical for reproductive success, as they potentially convey information related to species identity, sexual state, and location. Information on species identity could serve to reduce the occurrence of interspecific mating, lowering the reproductive costs associated with hybrid offspring (i.e. male sterility) (11).

How do females respond to male advertisement call? The male-female vocal interaction of *X. laevis* South Africa, one of the 29 species, have been well-studied and documented (12), offering researchers a good understanding of courtship interactions within the genus. Females,

when sexually receptive, respond with a vocalization called rapping, characterized by a series of fast pulses (13). The male and the female engage in vocal duets until the successful locating of each other and enter amplexus, the mating position. Rapping provides crucial auditory cues for males to locate the females in the murky and populous ponds in time for oviposition, the release of eggs by the females. Sexually-unreceptive females can also produce ticking, a type of release call to facilitate the exiting of amplexus (14).

However, apart from *X. laevis South Africa*, the duetting patterns of other species have not been previously reported. It remains unknown whether species-differences, in terms of spectral and temporal features, exist in female rapping. It's also unclear if further fine-scale coordination or synchrony in calling exists: whether different species of females respond to certain species-specific features in male calls. Given the diverse phenotypes of male advertisement call, it's reasonable to believe such divergent evolution is accompanied by co-evolution in female response, or otherwise the changes in signal would less likely to have persisted (15, 16).

Herein, we investigate the occurrence and acoustic features of male-female courtship vocalizations across species in the L clade (*X. laevis South Africa*, *X. laevis Malawi*, *X. petersii*, *X. victorinus*, *X. poweri*) of the *Xenopus* genus. Through recording vocal duets, we characterize female vocal responses to male calls. Through phonotaxis experiments, we compare differences in female movement responses when exposed to male calls between from the same species and from other species. We analyze these data to quantitatively determine the female responses to male advertisement calls. The findings are compared across species based on the molecular phylogeny of the genus that provides an evolutionary framework. These comparisons are then used to evaluate the evolutionary trajectories of both male calls and female preferences. The results support a working model in which sexual selection forces enact through the paradigm of vocal turn taking.

2. Materials and Methods

2.1 Laboratory Animal

Animals used for the experiments were all sexually mature clawed frogs from the sub-genus *Xenopus*. The animals were either purchased from commercial suppliers (Xenopus Express, Xenopus One, Nasco, or Avifauna) or the *Xenopus* colony at Columbia University's aquatic animal facilities as described in Kwong-Brown et al. (8). The specific species and

populations are listed as follows: *X. laevis South Africa*, *X. laevis Malawi*, *X. petersii*, *X. victorianus*, and *X. poweri*. The detailed geographic locations where the species were originally obtained are described in reference (9). The frogs were maintained in polycarbonate tanks containing dechlorinated water at pH7 and on a 12-12 light-dark cycle (lights on from 8:00 to 20:00). Temperature was maintained at 20°C. Animals were fed frog brittle (Nasco; Ft. Atkinson, WI, USA) 3 times a week, and the water was changed twice per week. All animal handling procedures were in accordance with guidelines established by the Columbia University Institutional Animal Care and Use Committee (IACUC).

2.2 Recording of vocal duets

For the recording of male-female vocalization, sexually mature female and male *Xenopus* were injected with human chorionic gonadotropin (hCG; 10,000 IU, Merck & Co. Inc.) before experiments to induce receptiveness. The amount (mL) of hCG injected is calculated by multiplying 0.05 by the mass of the individual (g). To observe female-male vocalization behavior, a female and a male were placed on either side of an opaque but acoustically transparent barrier in a glass aquarium tank (50 × 25 × 30 cm; L × W × H; water depth = 23 cm; volume = 46 L). A hydrophone (High Tech, Gulfport, MI, USA; output sensitivity -164.5 dB at 1 V/μPa, frequency sensitivity 0.015–10 kHz) was mounted 0.05 m below the water surface on the female side of the barrier to record vocalization. Calls were recorded at a 44.1 kHz sampling rate via an Audiombox 1818VSL (PreSonus, Inc., CA, USA) and processed using digital audio workstation software Reaper (Cockos, Inc., San Francisco, CA, USA). Additional barriers were used to restrict frogs' movement so that the maximum distance between the hydrophone and the vocalizing frogs is 30 cm.

Each male-female pair goes through two stages of recording. The purpose of the first stage is to observe behaviors between receptive males and receptive females to record female rapping. Recordings were conducted overnight, typically from 6:00 pm to noon the following day, collecting audio data for a total duration of 18 hours. The purpose of the second stage is to record vocalizations between receptive males and unreceptive females to record female ticking. Soon after the stopping of the recordings at noon, the male frogs were put on the same side of the barrier together with the now unreceptive female frogs. New recordings would continue for a duration of 5 hours (noon – 5:00 pm, on the same day). It was assumed that the effects of hCG

had declined after 18 hours, and therefore the females would no longer be receptive to male courtship. All recordings were done in anechoic chambers to prevent noise interference.

For sample size, we obtained valid recordings on 7 pairs of *X. laevis* South Africa, 3 pairs of *X. laevis* Malawi, 15 pairs of *X. petersii*, 9 pairs of *X. victorinus*, and 10 pairs of *X. poweri*.

2.3 Phonotaxis Experiment

To determine female movement responses to male calls, phonotaxis experiments were conducted. Sexually mature female *Xenopus* were injected with hCG and each placed in one 77 × 32 × 32 cm glass aquarium tank (L × W × H; water depth = 23 cm; volume = 91 L). Two plexiglass panels (31 × 31 cm) were placed inside a tank 5 cm from the either W × H surface of the tank perpendicular to the 77 cm sides. Two transducers (DAEX25 Sound Exciter, Dayton Audio Inc., OH, USA) were placed on each of the plexiglass panels at a height of 8 cm from the tank bottom. When tanks are filled with water, the transducers will be below water level. Each transducer is connected to a Lepai LP-2020TI Digital Hi-Fi Stereo Audio Amplifier, which is connected to a computer playing male calling segments. Vibrating transducers turn plexiglass surfaces into speaker systems to broadcast sound underwater. Stimulus tapes include male calls from *X. laevis* South Africa and *X. petersii*, as well as white noise for control. Tapes were programmed to play from either side's transducer alternatingly, and observations focused on whether female frogs show more preferences in moving towards calling from the same species over other species. Female *Xenopus* used for phonotaxis experiments include individuals from *X. laevis* South Africa, *X. petersii*, F1 hybrids between the previous two, and *X. victorinus*.

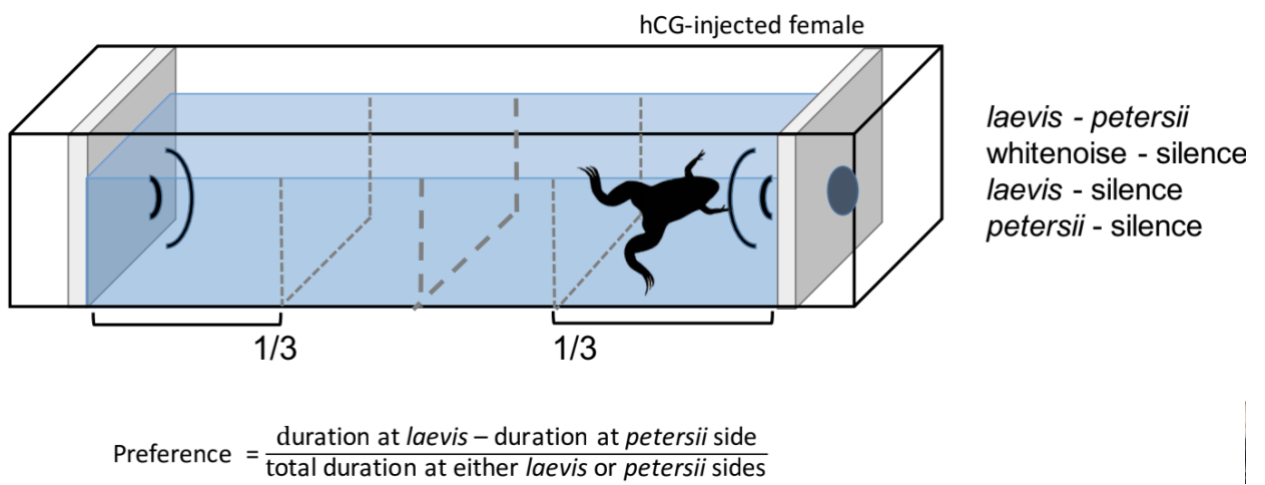


Figure 1. Set-up for phonotaxis experiments

2.4 Computational Analyses

To quantitatively measure female vocal responses to male calls, computational pipelines were designed to first identify bouts of calling using a Hidden Markov Model, with the hidden states being “yes call” and “no call” and observed states being the different amplitudes. Original recordings were first smoothed using the Hilbert transform and Gaussian filter, and the mean and standard deviation of all amplitudes were calculated. The signals were normalized by calculating the z score at each point. Based on the assumption that most of the recording would consist of silence, we set the threshold amplitude to be counted as calling to be $z \text{ score} = 2$ ($z \text{ score} > 2$ would be classified as “yes call” state). Using logistic regression ($\mu = 2, s = 1$), we calculated the predicted likelihood of “yes call” states at each datapoint. We then used this as a parameter for the Viterbi discriminative algorithm to calculate the sequences of most likely states, which were then used to remove silent segments from the recordings to increase the efficiency of analysis. For the next steps, we used Fourier transform to separate the bouts recordings to those of males and females, based on the fact that females call at frequencies distinctly lower than males.

For the pure male call segments, we implemented another Hidden Markov Model to perform call tagging, identifying call types (burst, click, fast trill, or slow trill) in the recordings. With this, we run association analyses on female calling time and male call types to identify whether females were preferring and responding to certain male call types. Subsequently, we cross-referenced the male call segments to determine whether certain call types are species-specific.

Taking the pure female call segments, we also investigate into the species-specificity of female calls by counting the Inter-pulse Interval (IPI) of female rapping and ticking. The algorithm finds intervals generated by 3 or more calls within 2 seconds, and removes outliers that lie 3 standard deviations more beyond the mean interval lengths.

2.5 Software Availability

All computational analyses were conducted using python 3.7, leveraging the following open-source frameworks, scientific computing libraries, and tools: NumPy (17, 18), SciPy (19), Pandas (20), Seaborn (21), Matplotlib (22, 23), PyWavelets (24), librosa (25). Customized code

is available at Github repository accessible with the link here.
(https://github.com/jamespan1118/Xenopus_signal_processing).

3. Results

3.1 Species-specificity of Female Vocalizations

While the species-specificity of male advertisement calls has been intensely studied, there are to date no reports on whether female calls differ between species. We investigate into this by referencing the two factors that differentiate male calls between species: spectral features and temporal patterns.

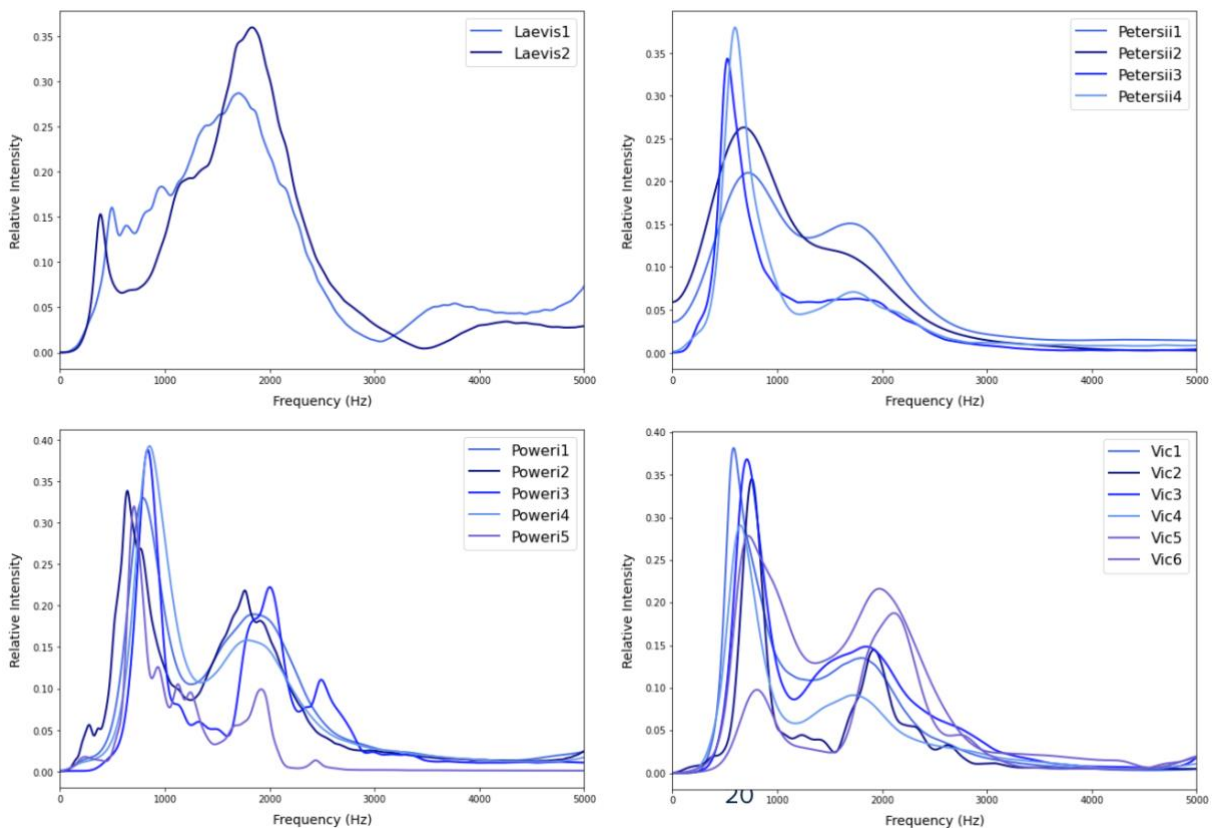


Figure 2. Fast-Fourier transform (FFT) analyzes of female rapping across species

For spectral features, it was found that the female call frequency ranges of *X. petersii*, *X. victorinus*, and *X. poweri* are similar, all revealing a relatively broad frequency distribution

from 500 Hz to 1200 Hz. Female *X. laevis South Africa* calls differ from the others by extending into higher frequency range, showing another broad peak at from 1000 Hz to 2500 Hz.

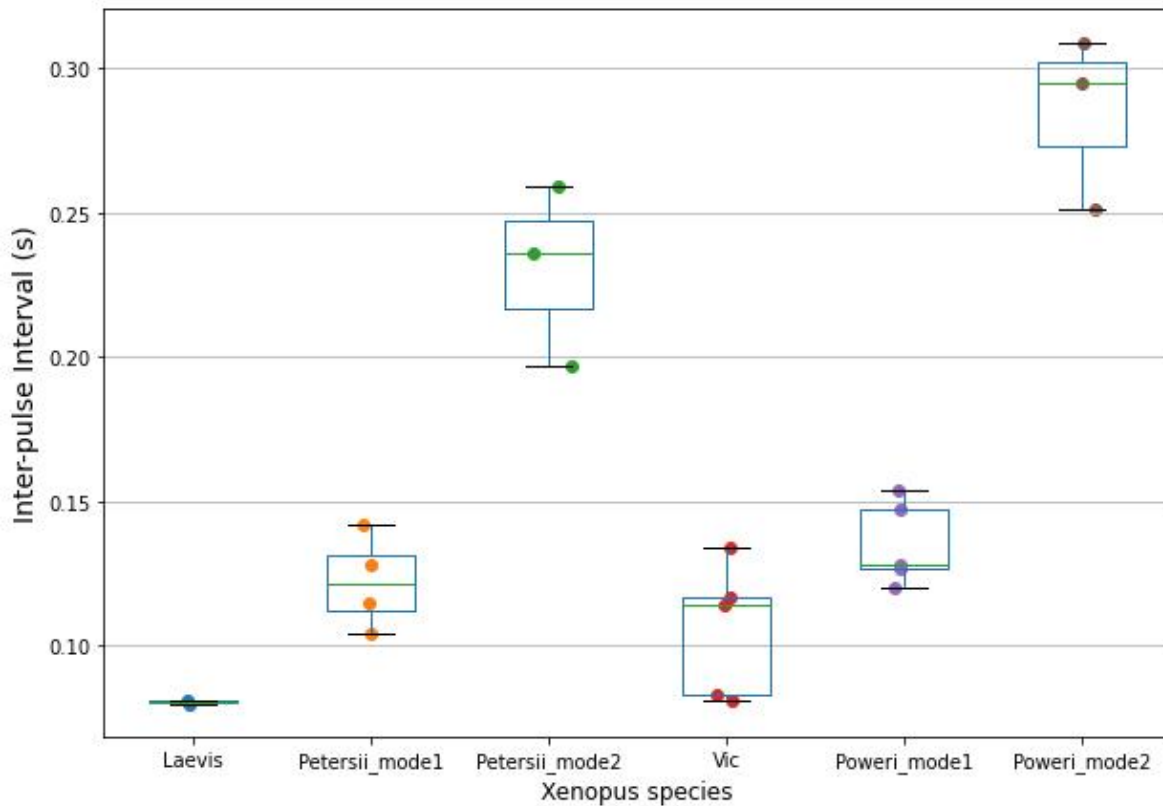


Figure 3. Inter-pulse Intervals (IPI) of female rapping across species

For temporal patterns of rapping, evidence of species-specificity is observed. *X. petersii* and *X. poweri* displayed bimodal patterns, while *X. victorianus* and *X. laevis South Africa* showed unimodal patterns. The lower modes IPI of *X. petersii*, *X. victorianus*, and *X. poweri* show overlapping trends, while the modes of *X. laevis South Africa* are lower than the others. The latter finding agrees well with the molecular phylogeny of the genus presented by Evans et al., which indicates that *X. laevis South Africa* is the most genetically distant from other species in the L clade (6). As for the bimodal distributions found in *X. petersii* and *X. poweri*, two species that are not genetically the most closely related, the origins could have two explanations. The first explanation is that the ancestral state was unimodal rapping, and *X. petersii* and *X. poweri* acquired bimodal rapping independently. The second explanation is that the ancestral state of bimodal rapping was lost in *X. laevis South Africa* and *X. victorianus* in evolution.

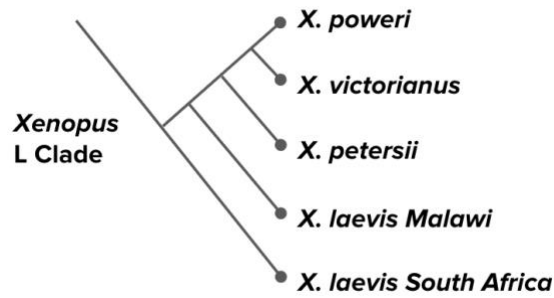


Figure 4. Phylogenetic relationship of *Xenopus* species (L Clade) in this study

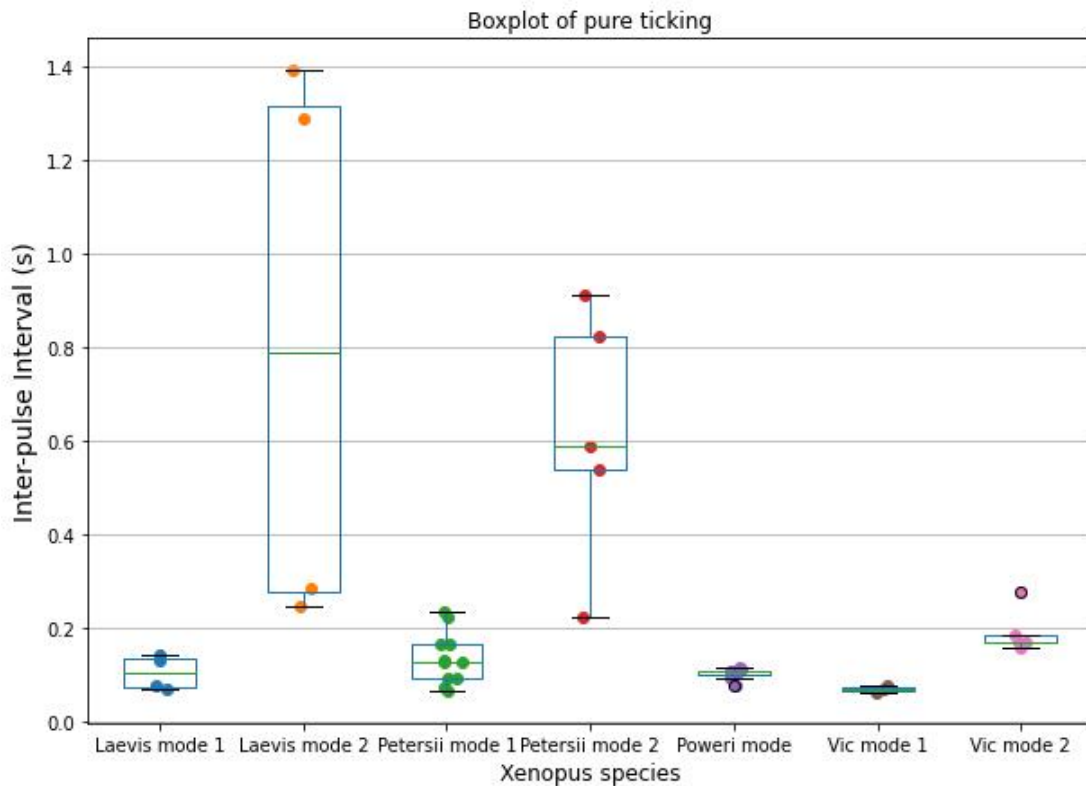


Figure 5. Inter-pulse Intervals (IPI) of female ticking across species

For temporal patterns of female ticking, bimodal distributions are found in *X. laevis South Africa*, *X. petersii*, *X. victorians*, while a unimodal distribution is found in *X. poweri*. This can also be explained by the molecular phylogeny in which *X. poweri* is genetically distant from the other three species. It's reasonably suspected that bimodal was the ancestral phenotype and was lost in evolution by *X. poweri*.

3.2 Female Movement Response to Male Calls

Results from the phonotaxis experiments are shown below. The preference of each female individual was calculated with a preference score formula: $\text{score} = (\text{duration on the } X. \textit{laevis} \textit{ South Africa} \textit{ side} - \text{duration on the } X. \textit{petersii} \textit{ side}) / \text{total duration}$. *X. laevis South Africa* females have a strong preference for the advertisement calls of *X. laevis South Africa* males, while *X. petersii* females doesn't show clear preferences. We also investigate the relationship between preference and time. From the Figure 7 below, no relationship can be identified. There are no temporal patterns in which preference change according to time.

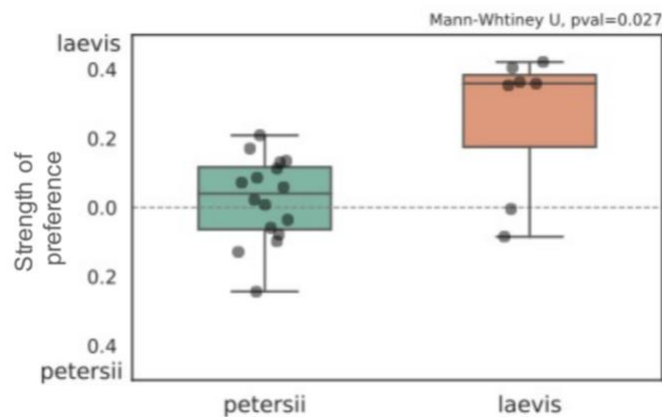


Figure 6. Box-plot showing the preference scores of *X. laevis South Africa* and *X. petersii* female individuals for recordings of male advertisement calls

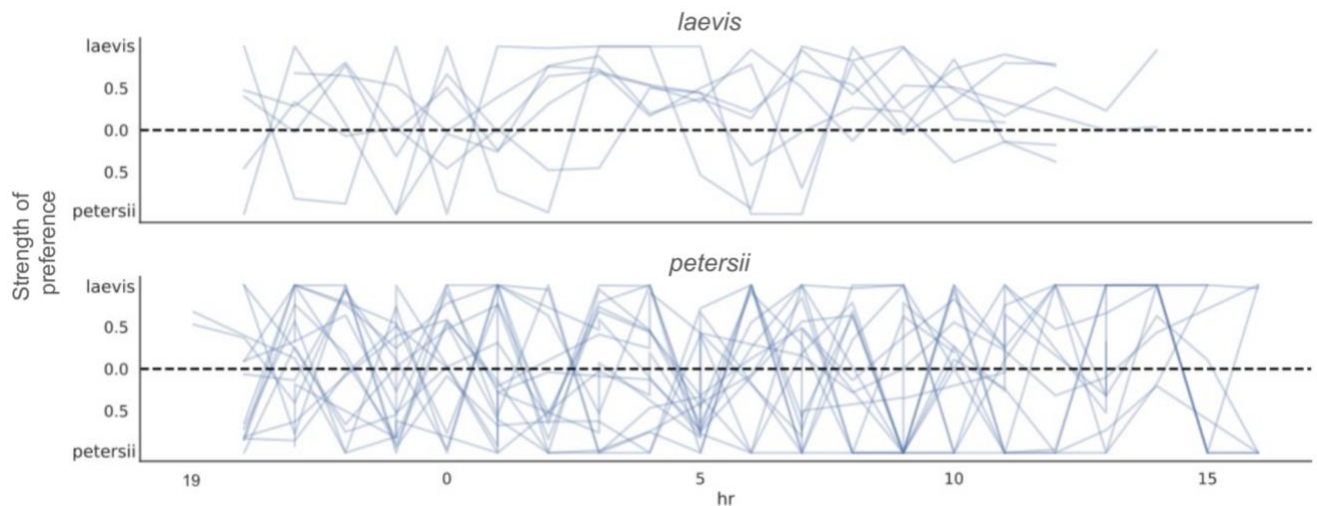


Figure 7. Relationship between preference score and time for *X. laevis South Africa* and *X. petersii* females

4. Discussion

Distinct Species: X. laevis South Africa

Quantitatively characterizing the courtship vocalization of difference species in the L clade, we found evidence that *X. laevis South Africa* is a more distinct species compared to others. The female rapping of *X. laevis South Africa* shows clear bimodal spectral patterns, while those of other species are more unimodal. What's also distinct is the inter-pulse interval of *X. laevis South Africa*, which is clearly unimodal and significantly lower than other species. This means that *X. laevis South Africa* has a faster and more consistent rate of rapping. While this agrees well with the phylogeny of *Xenopus* (6), it's also interesting to ask why the interspecies difference between *X. laevis South Africa* and other species is greater than others. Could it be related to the habitat of the species? *X. laevis South Africa* is from South Africa, while *X. victorinus*, *X. poweri*, and *X. petersii* are from central parts of Africa like Congo and Sudan. Environment in southern Africa, including drying weather, might be selecting for these distinct traits in *X. laevis South Africa*. If such is the case, then *X. gilli*, also native to South Africa (26), should exhibit similar calling characteristics to *X. laevis South Africa*. Further investigation can be conducted by recording the courtship vocalization of *X. gilli* to determine whether these differences are the result of habitats.

Preferences of female to same species male calling

For movement response of females to male advertisement calls, there's no real strong preference from the *X. petersii*. This observation could be explained from a biological perspective, which suggests that plausibly selection for species auditory cues is specific to *X. laevis South Africa* females and is not reciprocated in *X. petersii* females. The selection by *X. laevis South Africa* females is what's mainly responsible for maintaining the species boundaries between *X. laevis South Africa* and other species. Yet, the observation might also be attributed to confounders including sexual receptivity, effective sound decay, hearing loss due to age, as well as differences in species strain. In the event that the *X. petersii* females used for experiments experience hearing loss due to age or they are from a different strain than *X. petersii* males that was used to record the advertisement call playback, the observation that *X. petersii* females doesn't show movement preference is not unexpected. More importantly, during the experiments it was observed that most *X. petersii* females did not lay eggs while all *X. laevis South Africa* laid

eggs with the exception of the two outliers. As egg-laying is a good indicator of sexual receptivity, it's reasonable to suspect that the *X. petersii* females used for experiments aren't in their most reproductive states. To further examine this, we have ordered more female individuals of *X. laevis South Africa* and *X. petersii* directly from The National Xenopus Resource (NXR) at the Marine Biological Laboratory. (MBL) with the hope of eliminating confounding variables. If the future results are still consistent with the previous, we should be able to conclude that female movement preference to advertisement calls from same-species males is specific to *X. laevis South Africa*.

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