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Sex differences and endocrine regulation of auditory-evoked, neural responses in African clawed frogs (*Xenopus*)

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Abstract

Mating depends on the accurate detection of signals that convey species identity and reproductive state. In African clawed frogs, *Xenopus*, this information is conveyed by vocal signals that differ in temporal patterns and spectral features between sexes and across species. We characterized spectral sensitivity using auditory evoked potentials (AEPs), commonly known as the auditory brainstem response, in males and females of four *Xenopus* species. In female *X. amieti*, *X. petersii*, and *X. laevis*, peripheral auditory sensitivity to their species own dyad- two, species-specific dominant frequencies in the male advertisement call – is enhanced relative to males. Males were most sensitive to lower frequencies including those in the male-directed release calls. Frequency sensitivity was influenced by endocrine state; ovariectomized females had male-like auditory tuning while dihydrotestosterone-treated, ovariectomized females maintained female-like tuning. Thus adult, female *Xenopus* demonstrate an endocrine-dependent sensitivity to the spectral features of conspecific male advertisement calls that could facilitate mating. *Xenopus* AEPs resemble those of other species in stimulus and level dependence, and in sensitivity to anesthetic (MS-222). AEPs were correlated with body size and sex within some species. A frequency following response, probably encoded by the amphibian papilla, might facilitate dyad source localization via interaural time differences.

Keywords

Xenopus; ABR; matched-filter; sex difference; androgen

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Introduction

In many species, acoustic signals serve as the primary means of social communication. Enhanced neural responses to vocal features can be demonstrated in higher auditory regions such as the midbrain in terrestrial frogs (Narins and Capranica, 1980; Edwards et al. 2002; Hoke et al. 2004) and in birds and mammals (Bauer et al. 2002; Klug et al. 2002; Portfors et al. 2009, Woolley and Portfors, 2013). The significance of acoustic features can also differ for males and females accompanied by sex differences in sensitivity of the auditory periphery and midbrain (Narins and Capranica, 1976; 1980; Shen et al. 2011). Matching peripheral auditory processing to informative acoustic features- a hypothesis explored in this study- could facilitate signal reception from a distant source or in noisy environments (Frishkopf et al. 1968; Narins and Capranica 1976; Sisneros et al. 2004; Moreno-Gomez et al. 2013; Simmons 2013).

African clawed frogs (*Xenopus*) are nocturnal, aquatic anurans whose social communication relies largely on producing and responding to vocal signals (Vigny 1979; Zornik and Kelley 2011). Spectral and temporal features of vocalizations convey species identity, sex and reproductive state (Tobias et al. 2011; 2014). Here we examine frequency-specific neural responses in four species: *X. amieti*, *X. petersii*, *X. laevis*, and *X. borealis*. In both sexes, the fundamental vocal unit is a sound pulse produced by the larynx (Tobias et al. 2011). The sound pulses in male advertisement calls are composed of spectral dyads: two, simultaneous dominant frequencies (DFs) referred to here as the species own dyad (SOD) (Fig. 1; Wetzel and Kelley, 1983; Tobias et al. 2011). Male advertisement calls are high intensity, long-range signals that attract sexually receptive females (Picker 1983; Yager 1992). In contrast, release calls, evoked when a male or an unreceptive female is clasped by a receptive male, are low intensity and close range (Tobias et al. 2014). Release calls contain a single DF that is similar across species (Tobias et al. 2014), thus spectral cues alone cannot convey information on sex and reproductive state.

We examined the sensitivity of the auditory periphery in *Xenopus* using a non-invasive approach: measuring auditory-evoked potentials (AEPs) from surface electrodes positioned on the head. This approach has been used in several anuran species (Katbamna et al. 2006a; Katbamna et al. 2006b; Brandt et al. 2008; Schrode et al. 2014) and is usually referred to as the auditory brainstem response (ABR). While the timing and quality of the signals suggest that these field potentials represent early stages (VIIIth nerve and hindbrain) in auditory processing (Seaman 1991; Hall 2007), the source of anuran AEPs has not yet been disambiguated in terms of contributions of activity of inner ear hair cells, VIIth nerve afferents and CNS auditory nuclei. We thus adopt the more general term AEP to refer to the sound-induced potentials recorded here.

Previous studies have suggested that the filtering properties of the anuran auditory system are matched to informative spectral features of calls (reviewed in Simmons 2013). In addition, while a variety of evidence suggests that gonadal steroids influence auditory-based social behavior in frogs, little is known about their activational effects on auditory processing (reviewed in Arch and Narins 2009). Frogs also exhibit size-related differences

in auditory sensitivity and behavioral responses to sound (Zakon and Wilczynski 1988; Keddy-Hector et al. 1992, Fox 1995; Meenderink et al. 2010), and peripheral auditory sensitivity can be shaped by the mechanical properties of the head and ear (Pinder and Palmer 1983; Smotherman and Narins 2000; Schoffelen 2009). Here, we also compared AEP recordings in the sexes to determine if the larger size of females contributes to auditory responses.

The sound pulses in *Xenopus* male advertisement calls are dyads composed of two DFs (*e.g.*, Fig. 1). Two tones presented simultaneously interact to generate a beat frequency equal to the higher frequency minus the lower. In mammals, sensitivity to these beat frequencies can be measured in the cochlea (Ruggero 1992) and perception is influenced by the frequencies and amplitudes of the tones used to generate them (Humes 1979). Behavioral (Simmons 1988; Hainfeld 1996) and electrophysiological (Capranica and Moffet 1980; Corwin et al. 1982; Klump et al. 2004) evidence in frogs has shown that many species are sensitive to beat frequencies, envelope modulations, or harmonic structure of acoustic stimuli. In anurans, two sensory epithelia transduce sound: lower frequencies (~0.1 to 1 kHz) are represented in the amphibian papilla and higher frequencies (~1 – 4 kHz) in the basilar papilla (Narins and Capranica 1980; Lewis et al. 1982). In many species of *Xenopus*, the beat frequencies created by DF2-DF1 of the advertisement call should be less than 1000 Hz, within the typical frequency range of the amphibian papilla (Lewis et al. 1982). We thus also asked if *Xenopus* are also sensitive to beats by examining the frequency following response (FFR), a phase locked neural response to periodic stimuli thought to be involved in processing complex sounds (Skoe and Kraus 2010). The FFR exhibits plasticity in response to experience with music and speech in humans (Krishnan and Grandour 2009; Krishnan et al. 2010; Bidelman 2013) and might exhibit plasticity in response to endocrine state. We thus asked whether the AEP could contribute to processing harmonic vocal signals in female *Xenopus* under different hormonal conditions.

Materials and Methods

Animal care and maintenance

Animals were housed and treated in accordance with Columbia University's IACUC requirements. Frogs were obtained from *Xenopus* Express (Brooksville, FL, USA), Nasco (Fort Atkinson, WI, USA), or were lab bred. The range and mean of [(male), (female)] body masses in grams were: *X. laevis* [(32 – 63, mean 50, n = 11), (48 – 177, mean 118, n = 22)]; *X. amieti* [(3 – 7, mean 5, n = 10), (8 – 14, mean 10, n = 14)]; *X. borealis* [(14 – 22, mean 18, n = 11), (17 – 45, mean 27, n = 11)]; *X. petersii* (formerly *X. laevis* Congo; Furman *et al.* 2015) [(12 – 18, mean 15, n = 11), (15 – 25, mean 23, n = 14)]. Frogs were housed in polycarbonate aquaria under a 12/12 light-dark cycle and fed Nasco frog brittle. Aquarium water was changed twice weekly.

A group of adult *X. laevis* females were ovariectomized (OVX; mass range 41–101 g, mean 69 g, n = 15) under tricane methanesulfonate (MS222; Sigma Aldrich, St. Louis, MO, USA) anesthesia. The ovaries and fat bodies were removed through an incision in the abdomen and blood vessels were closed with cautery. A second group (mass range 48 – 98 g, mean 78 g, n

= 9) underwent ovariectomy and was implanted with a compressed pellet (Parr Instrument Company Pellet Press; Moline, IL, USA) of 10 mg of dihydrotestosterone (DHT; Sigma Aldrich, St. Louis, MO, USA) in the dorsal lymph sac at the time of surgery. The wound was sutured and the animals recovered for 6 – 8 weeks prior to recording AEPs.

Preparation for Recording

Animals were immobilized with an injection of d-tubocurare (Sigma-Aldrich; St. Louis, MO, USA) into the dorsal lymph sac (3 µg/kg *X. amieti*; 8 µg/kg *X. petersii*; 10 µg/kg *X. borealis*, *X. laevis*). Frogs were maintained at ambient temperature (22.5 – 24 °C) and were draped with a wet Kimwipe (Kimberly-Clark Global Sales, LLC; Roswell, GA, USA) to maintain skin moisture. Oxygen levels were maintained via cutaneous respiration and air lung reserves. Animals were pre-treated with subcutaneous lidocaine prior to AEP electrode insertion. If an animal regained motility during recording, supplemental curare (3 µg/kg; Sigma-Aldrich; St. Louis, MO, USA) was administered via the dorsal lymph sac followed by a 15 – 30 minute pause in recording. After recording, animals were allowed to recover on a float in their home tank, while draped in a wet Kimwipe to maintain skin moisture.

AEP Procedures and Equipment

We recorded auditory evoked potentials in a sound-attenuated chamber (2 m × 1.6 m × 2 m; Industrial Acoustics Company, Inc., New York, NY, USA). Three, platinum, 30-gauge needle electrodes (Grass Instruments, West Warwick, RI, USA) were inserted beneath the dermis: 1) non-inverting - above the foramen magnum, 2) inverting - posterior to the tympanic disk, 3) ground – dorsal, mid-anterior/posterior. Frogs were stimulated with airborne sound through a closed-field coupler (Christensen-Dalsgaard et al. 2012) that was sealed around the ipsilateral tympanum using ear mold compound (Gold Velvet II; All-American Mold Laboratories, Oklahoma City, OK, USA). The coupler was composed of a speaker (Yuin PK2; Yuin Corporation, China) epoxied to an otoscope tip containing a microphone for calibration (FG-23742-D36; Knowles, Itasca, IL, USA). After sealing the coupler around the tympanic disk, the speaker was calibrated to a 300–7000 Hz frequency sweep using BioSigRZ software (TDT, Gainesville, FL, USA). Microphone sensitivity and calibration were confirmed before and after these experiments using a PCB CAL200 Precision Acoustic Calibrator (Provo, UT, U.S.A.) and model 377B01 condenser microphone (PCB Piezotronics, Inc., Depew, NY, USA). AEPs were acquired using a 10 Hz high-pass filter, a 3000 Hz low-pass filter, and a 60 Hz notch filter.

We used the closed-field coupler to present auditory stimuli because it reliably delivers specific frequencies directly to the ear. The frequencies used in this study are often distorted when played underwater in the relatively small tanks that are compatible with recording auditory evoked potentials in the laboratory (Akamatsu et al. 2002). Audiograms obtained using the closed-field coupler were consistent with audiograms obtained underwater using behavioral conditioning, including enhanced resolution of frequencies near the DFs of the advertisement call (Elephant et al. 2000). The closed-field coupler stimulates the ear and does not mimic the effects of free-field sounds that are influenced by other parts of the frog's body such as the mouth or lungs (Pinder and Palmer 1984; Aertsen et al. 1986; Narins

et al. 1988; Jørgensen et al. 1991). Thus, enhanced sensitivity to frequencies outside of the range of communication vocalizations (3400 Hz; Elepfandt et al. 2000) that have been observed underwater may not be observed with closed coupler stimulation. The spectral sensitivities observed with the closed coupler stimulation technique should also be observed underwater, however.

Stimuli

Stimulus presentation, response acquisition and data storage were conducted with Tucker Davis Technologies (TDT; Gainesville, FL, USA) system III (RZ6 with an RA4PA Medusa preamp with RA4LI headstage) and a PC running SigGenRZ and BioSigRZ software (TDT, Gainesville, FL, USA). AEPs were the average neural response to 512 presentations of each stimulus, alternating in phase by 180 degrees. The inter-stimulus interval was 21 ms.

Clicks

Clicks were square wave pulses, 0.1 ms in duration that produced broadband frequency stimulation up to approximately 60 kHz. Clicks were presented at high intensity (111 dB) to stimulate as many auditory cells as possible. Measuring the amplitude of the click-evoked response (Fig. 2a) allowed us to measure and track general auditory responsiveness across the recording period. Responses to clicks were recorded at four time points over the course of each recording session. In curarized frogs, click amplitudes did not differ significantly from the beginning to the end of the experiment (paired t-tests, all $p > 0.05$). Across the population, click amplitude decreased by an average of 3% from the beginning to the end of the experiment.

Tones

Audiograms were generated by recording AEPs to pure tones (10 ms duration, 5 ms rise/fall time, cosine ramp) at approximately 250 Hz steps between 1000 and 4000 Hz, including the DFs of the SOD. To compare frequency sensitivity between sexes, we normalized each audiogram by setting its lowest threshold to 0 dB.

Two-tone dyads

Two-tone (dyad) stimuli (10 ms duration, 5 ms rise/fall time, cosine ramp) were generated and power-matched (root mean square) using customized MATLAB scripts (MathWorks; Natick, MA, USA). Stimuli were 10 ms in duration in order to mimic the sustained portion of the frogs' sound pulse (Vignal and Kelley 2007) and to allow for adequate frequency information in multi-frequency stimuli. Analysis of spectrograms confirmed well-defined frequency peaks > 50 dB above maximum background levels. Dyads were presented at 100 dB.

Each species has a unique dyad (DF1, the lower frequency and DF2) and DF2:DF1 ratio (Tobias et al. 2011). The SODs and dyad frequency ratios of the species we studied are: *X. amieti* [2040 Hz, 2710 Hz], 1.33; *X. borealis* [1250 Hz, 2500 Hz], 2.0; *X. petersii* [2110 Hz, 2580 Hz], 1.22; *X. laevis* [1890 Hz, 2160 Hz], 1.14 (Fig. 1d). These values were obtained

from *in vivo* recordings, as described previously (Tobias et al. 2011) and updated with additional calls analyzed by M. Tobias and U. Kwong-Brown.

Two-tone stimuli were presented to frogs while recording AEPs in order to investigate sensitivity to dyad frequencies and frequency ratio. The two tones in our synthetic stimuli were of equal intensity. To test selectivity for frequency, the SOD -[DF1, DF2]- was presented along with dyads in which one of the tones was modified. In one manipulation-[DF1, Variable]- the higher frequency was changed to create dyads with frequency ratios of 1.14, 1.22, 1.33, 1.39, 1.5, 1.6, 1.67, 1.78, 1.88, and 2.0. In the other manipulation-[Variable, DF2]- the lower frequency was changed to create frequency ratios of 1.14, 1.22, 1.33, 1.39, 1.88, 2.0. Frequency ratios (1.14, 1.22, 1.33, 1.5, 2.0) represent those used by various *Xenopus* species. The additional ratios were from the Western musical scale. More frequency ratios were tested in the first manipulation because DF1 tends to be more prominent in male vocalizations (Tobias et al. 2011).

To examine frequency ratio sensitivity specifically, two additional dyad sets were generated for each species using frequencies not found in male vocalizations. Tones with frequencies near DFs were used as the higher tone: *X. amieti*, 2250 Hz, 2500 Hz; *X. borealis* and *X. petersii*, 2000 Hz, 2250 Hz; *X. laevis*, 2000 Hz, 2500 Hz. The frequency of the lower tone was changed to create ratios of 1.14, 1.22, 1.33, 1.39, 1.88, and 2.0.

For each species, a single series of all 27 dyads was generated. Stimuli were ordered to maximize the frequency disparity between temporally adjacent dyads. To estimate thresholds, each dyad was presented at three intensities, in 10 dB steps. After initial threshold estimation with AEPs recorded at the 3 intensities, the series order was reversed and additional AEPs were collected in order to measure threshold at a 5 dB intensity resolution. In 30% of frogs, the dyad series order was reversed for the initial collection to test for stimulus order effects. No presentation order-related differences in threshold were observed.

Band-limited masker

Two simultaneous tones produce a beat frequency via amplitude modulation of the stimulus envelope at the rate of the difference between the two frequencies. A beat frequency less than 1000 Hz should specifically stimulate one of the two peripheral auditory organs: the amphibian papilla (Lewis et al. 1982). Since the SOD beat frequency, DF2-DF1, should be less than 1000 Hz for most *Xenopus* (Tobias et al. 2011), dyads might stimulate both the amphibian and the basilar papillae producing to enhanced sensitivity to the SOD. We thus repeated these experiments while playing a band-limited masker to occlude the influence of the amphibian papilla. By playing continuous 100–800 Hz noise during recording, we asynchronously activated cells in the amphibian papillae thus averaging out their effect on the AEP and allowing us to specifically determine the response characteristics of the basilar papilla.

In 5 female *X. laevis*, a continuous, band-limited masker was played during dyad presentation. The masker was presented to the ipsilateral ear through a second earbud

mounted to Y-shaped plastic tube in a modified version of the apparatus described in section 2.2. To set the intensity of the masker, the band was set at 100–5000 Hz and intensity was increased until the AEP peak height to a 111 dB SPL click was decreased by >75%. When played with dyads, the masker was band limited to 100–800 Hz.

AEP analysis

AEPs were acquired using a 10 Hz high-pass filter, a 3000 Hz low-pass filter, and a 60 Hz notch filter. To facilitate quantitative analysis and threshold determination after data collection, an additional 1500 Hz low pass filter was applied to increase the signal to noise ratio. AEP peak to peak frequencies (first positive peak to second positive peak, P1 to P2) ranged from approximately 200–500 Hz near threshold to 1000 Hz at high stimulus intensities, which is consistent with those observed in other studies in *Xenopus* (Katbamna et al. 2006a; Katbamna et al. 2006b). For peak height assessment, each AEP was frame-shifted on the y-axis so that the average pre-response (0 – 2 ms) value was zero to account for individual variation in offset. Peak latency was measured from the time of stimulus onset; the speaker was, on average, 1.5 cm from the ear resulting in a negligible 0.04 ms delay between stimulus onset and vibration of the tympanic disk.

The response threshold for each stimulus was estimated as the average of the lowest intensity evoking a significant response super-threshold and highest intensity that failed to evoke responses. Significant response waveforms met three criteria: 1) amplitude of the first positive (P1) or negative (N1) peak was at least 2 standard deviations above noise levels measured during the 3 ms pre-response period; P1 occurred from 3 – 8 ms, N1 from 4 – 9 ms; 2) P1 or N1 amplitude exceeded the maximum noise amplitude during the pre-response period; and 3) P1 amplitude exceeded average noise levels over the recording session. Criteria 1 and 3 were adjusted by increasing stringency but held constant for each individual in order to best fit species-typical response amplitude and individual signal-to-noise ratio. Criterion 1 was adjusted by increasing the number of standard deviations between 2 and 3 and Criterion 3 was adjusted by increasing the threshold above average noise level.

Statistical analysis

For each individual in this study, threshold was estimated for all dyads. Thresholds were normally distributed (Shapiro *et al.* 1968). The multiple frequency manipulations used to generate dyads prevented dyad identity from being treated as a continuous variable. Dyad identity was instead treated as a categorical variable in a repeated measures analysis of variance (ANOVA). A Dunnett's multiple comparison *post hoc* test was used to compare the thresholds for all other dyads to that of the SOD.

We reduced the dimensionality of the dyad variable by calculating the absolute frequency difference from SOD for each dyad. To calculate absolute frequency difference from SOD, we summed the absolute value of the minimum differences between a dyad's frequencies and those of the SOD. For example, the SOD for *X. laevis* is [1890 Hz, 2160 Hz]. The absolute frequency difference between the SOD and the dyad [2000 Hz, 2280 Hz] would be: $\text{abs}(1890 \text{ Hz} - 2000 \text{ Hz}) + \text{abs}(2160 \text{ Hz} - 2280 \text{ Hz}) = 230 \text{ Hz}$. Absolute frequency

difference as a continuous variable allowed us to examine the relationship between threshold and dyad disparity from SOD but was not used to determine which dyads had significantly higher thresholds than the SOD. Absolute frequency differences were not normally distributed so a Spearman rank order correlation analysis was used.

For audiograms, we examined the relationship between threshold and tone frequency in each species. Tone_frequency could be treated as a continuous variable. However, because of the complex, non-monotonic relationship between threshold and tone frequency, we treated tone_frequency as a categorical variable in a repeated measures ANOVA. Sex and sex*tone_frequency were incorporated into the model. Sex was significant where reported but sex*tone_frequency was not significant for any species.

For comparison of OVX, OVX+DHT, and intact female *X. laevis*, we used similar methods, replacing treatment with sex. To examine differences in histogram distributions we performed a one way ANOVA on thresholds by treatment.

To examine the effect of sex, species, and size on AEP amplitude we began with a forward selection, stepwise regression analysis. Sex, species, size, and all pairwise comparisons were included in the variables that could have been incorporated into the model. Other tests were used to evaluate hypotheses generated by the full model and explore potential species differences as noted.

Statistical analyses were performed with STATA (v9.0; StataCorp LP, TX, USA) and R (v3.1.3; R foundation, Austria) software.

Results

Stimulus effects on AEP waveform

AEPs in *Xenopus* were similar in shape and pattern to those reported for other frogs and classes of animals (Corwin et al. 1982; Britton-Powell et al. 2002; Katbamna et al. 2006a; Katbamna et al. 2006b; Schrode et al. 2014; Vélez et al. 2015). The AEP waveform is composed of positive and negative peaks; the shortest latency peaks identified as positive 1 (P1) and negative 1 (N1; Fig. 2). In general, P1 was the most prominent and AEP amplitude increased and latency decreased with increasing stimulus intensity. However, the overall shape of the AEP waveform was different for each stimulus. For example, AEPs evoked by clicks had one or two high amplitude peaks, while responses to tones and dyads were generally more variable in shape, with peaks of smaller amplitudes and longer latencies (Fig. 2b, c). The difference between click and tone AEPs is likely due to differences in the stimulus (Fig. 2). Clicks indiscriminately excite auditory neurons during a brief, 0.1 ms stimulus which leads to a large, brief field potential; tones excite a smaller population of auditory neurons that respond to that frequency and do so over a longer time scale.

We asked whether AEP waveforms were qualitatively different in response to different tone frequencies, particularly the DFs of the species advertisement call. We observed no difference after controlling for threshold. When presented at equal intensities with respect to threshold, DF and non-DF tones produced similar AEP waveforms (not shown).

Sex differences in dyad thresholds

In females, auditory thresholds for the SOD were lower than those of other dyads. Changing either DF1 or DF2 by as little as 200 Hz significantly increased thresholds (Fig. 3).

Threshold differences between stimuli that matched the SOD and stimuli that did not match the SOD were significant for the majority of comparisons: 89% in *X. amieti*, 70% in *X. petersii*, and 96% in *X. laevis* (Fig. 3c; Repeated Measures ANOVAs, Dunnett's multiple comparison post hoc, all $p < 0.05$). In males, AEP thresholds for the SOD were not lower than thresholds for other dyads (Fig. 3). Frequency shifts away from the SOD had more variable effects on thresholds and only in *X. laevis* did a frequency shift result in a higher threshold than the SOD (Fig. 3b). In both males and females, dyads composed of higher frequencies had higher thresholds; thresholds increased with the sum of the two frequencies in a dyad (Spearman; $\rho = 0.46$ males, 0.39 females, all $p < 0.001$; not shown).

We asked if females displayed peripheral auditory sensitivity to the frequency ratio used in the advertisement call of their own species. In female *X. amieti*, *X. laevis*, and *X. petersii*, as the absolute magnitude of the frequency shift away from the SOD increased, thresholds also increased (Fig. 4a; Spearman, all $\rho > 0.45$, all $p < 0.02$). Dyads sharing the species-specific ratio were similar to dyads with other ratios (Fig. 4b,c). A low threshold was only observed for dyad of the species own DF1 and DF2. Thus, female *Xenopus* are peripherally sensitive to the combination of DF1 and DF2 used in their conspecific male advertisement call but not to the species-typical frequency ratio when produced by non-species specific DFs.

While a similar pattern was observed in *X. borealis*, females AEPs showed no threshold differences between SOD and non-SOD stimuli (Figs. 3, 4). Because peaks in the frequency spectra of natural sounds at 2:1 ratios contribute to octave equivalence (sounds with 2:1 ratios are perceptually equivalent to each other even in non-human species; Wright et al. 2000), *X. borealis* females may perceive the SOD stimulus as a single frequency. In other frog species with a 2:1 call frequency ratio, there are minimal sex differences in auditory tuning (Schrode et al. 2014). In frogs that produce calls made up of only one frequency, adding tones to create 2:1 ratios (as well as other ratios used by *Xenopus*) had no effect on female preference (Gerhardt et al. 1990; Witte et al. 2001; Gerhardt et al. 2007; Gerhardt and Humfeld 2013), suggesting that some frogs are more tuned to frequency than frequency ratio for behavioral decision making.

Sex differences in tone thresholds

Sex- and endocrine-mediated differences in dyad sensitivity could reflect differences in tuning to individual frequencies. We measured thresholds to pure tones within the dyad frequency ranges of all four species, including the DFs of each species' SOD (Fig. 5). Thresholds for pure tones generally increased with frequency in both sexes and all species (Fig. 5; Spearman, all $\rho > 0.61$, $p < 0.001$). In *X. laevis*, *X. amieti*, and *X. petersii*, audiograms differed by sex (Repeated Measures ANOVAs; all $p < 0.02$). Male threshold minima occurred at or below 1500 Hz, frequencies in release calls (Tobias et al. 2014), and increased above 1500 Hz. Female threshold minima often included DF1 and DF2 of the

SOD. Only the *X. amieti* DF1 threshold was significantly different between sexes (Repeated measures ANOVA with Bonferroni post hoc $F_{(1,22)} = 8.86$, $p = 0.007$).

Endocrine signals influence female spectral sensitivity

In *Xenopus*, sexual differentiation of vocal motor circuitry relies on endocrine differences between the sexes (Kelley, 1986; Rhodes et al. 2007; Zornik and Kelley 2011). Ovarian estrogen derives from the aromatization of testosterone (Gruber et al. 2002); androgens are the primary ovarian steroids in *Xenopus* (Lutz et al. 2001). Because cell bodies in the acoustic ganglion express androgen receptor mRNA (Perez et al. 1996), we tested the hypothesis that female sensitivity to SOD frequencies depends on ovarian steroids. We compared AEP thresholds to tones and frequency dyads in ovariectomized (OVX), intact and OVX + dihydrotestosterone-treated females. Dihydrotestosterone (DHT) is a non-aromatizable androgen.

AEPs in OVX females were less sensitive to the SOD compared to intact controls (ANOVA, $F_{(2,81)} = 30.13$, $P < 0.001$, Intact versus OVX: Bonferroni post hoc $F = -5.09$, $p < 0.001$), resulting in male-like responses (Fig. 6A). The dyad threshold distribution in OVX females differed significantly from that of OVX+DHT females (OVX versus OVX+DHT: Bonferroni post hoc $F = -2.154$, $p = 0.005$) that retained a more female-like response profile (orange, Fig. 6A). The difference in threshold distribution between OVX+DHT and intact females (Intact versus OVX+DHT: ANOVA $F_{(2,81)} = 30.13$, $p < 0.001$, Bonferroni post hoc $F = 2.932$, $p < 0.001$) indicates that the selectivity of OVX+DHT females for the SOD was not as robust as in intact females. Thus DHT only partially maintains their sensitivity to the SOD.

Audiograms for individual frequencies were male-like in OVX females (Fig. 6B) and differed from audiograms of intact females (Repeated Measures ANOVA, $F_{(1, 350)} = 6.36$, $p = 0.01$), with a significant threshold increase at DF2 (Bonferroni, $F_{(1,35)} = 4.31$, $p = 0.04$). OVX+DHT female audiograms did not differ from those of intact females (Fig. 6B; Repeated Measures ANOVA). The greatest difference between OVX and OVX+DHT audiograms was the significantly greater sensitivity to DF2 after DHT treatment (Repeated Measures ANOVA, frequency*treatment interaction, $F_{(10,220)} = 1.93$, $p = 0.04$; Bonferroni post hoc $F_{(1,22)} = 6.65$, $p = 0.01$).

Dyad beat frequencies influenced AEP waveforms

The presentation of two tones simultaneously produces a beat frequency via amplitude modulation of the stimulus envelope at the rate of the difference between the two frequencies (Frequency 2 (F2) – Frequency 1 (F1); Fig. 2c, for example). The specific combination of two frequencies [F1, F2] in a dyad will generate characteristic beat frequencies for each. While not determined specifically for *Xenopus*, frequencies 1000 Hz and greater are thought to stimulate hair cells in the basilar papilla (Lewis et al. 1982) while those less than 1000 Hz stimulate the amphibian papilla. For most *Xenopus*, the SOD beat frequency, DF2-DF1, is less than 1000 Hz (Tobias et al. 2011). Thus, a dyad and its beat

frequency might stimulate both the amphibian and basilar papillae simultaneously, contributing to enhanced sensitivity to the SOD.

To investigate the effects of beat frequencies, we measured AEPs to dyads played at high intensity (100 dB), where the AEPs to dyads differed from AEPs to tones (Fig. 2b, c). The waterfall plot in Fig. 7a shows the averaged AEP waveforms to selected dyads for *X. borealis* females. As beat frequency increased from 364 Hz to 630 Hz, the frequency of AEP peaks increased, P1 latency decreased, and P1 peak height increased. At beat frequencies above 630 Hz, these qualities of the AEP waveform appeared to stabilize. There was no qualitative difference between the AEP waveform to the SOD (beat frequency 1250 Hz) and dyads with similar beat frequencies. There was also no effect of endocrine state on the FFR; OVX and OVX+DHT *X. laevis* females had FFRs that were indistinguishable from intact females and males (data not shown). In all species, males had FFRs similar to females, so the remaining analyses were grouped by species alone.

In all four species, the frequency of AEP peaks (P1, P2) followed the beat frequency of the dyad stimulus between approximately 200 and 700 Hz (Fig. 7b). Above beat frequencies of 700 Hz, the AEP peak frequencies were comparatively stable but less than their maximum. The change in peak frequency was correlated with a decrease in latency to P1. As beat frequencies increased to 700 Hz, latencies decreased until they stabilized at approximately 4 ms (Fig. 7c). P1 amplitude increased with beat frequency up to 600 Hz before stabilizing at a sub maximal value (Fig. 7d). Three of the species tested had SODs with beat frequencies less than 700 Hz, *X. amieti*, *X. laevis*, and *X. petersii* (Filled symbols, Fig. 7b–d). Among these, only the *X. petersii* SOD has a beat frequency (470 Hz) near the optimum for each of these categories, suggesting that the *Xenopus* frequency following response is not a specialization for communication within species, but is instead a generalized property of their peripheral auditory system.

To test the hypothesis that amphibian papilla is involved in the frequency following response to beat frequencies below 700 Hz, we repeated these experiments while playing a band-limited masker to occlude the influence of the amphibian papilla on the AEP. In 5 *X. laevis* females, we presented the dyad stimuli at 100 dB, and the dyad stimuli at 100 dB in the presence of a 100–800 Hz, band-limited masker. In this subset of animals, the response to the dyads alone (open symbols) was similar to the population (compare Fig. 7 b to e, c to f, d to g). When compared to the intra-animal controls, the AEPs to dyads with a masker (filled symbols) showed a clear loss of the frequency following response (Fig. 7e) and the latencies were less variable (Fig. 7f), though the relative peak amplitudes were qualitatively similar (Fig. 7g). These results suggest that the sensory organ responsible for transducing low frequency sounds, the amphibian papilla, is involved in the frequency following response.

In humans, the FFR correlates with the perception of consonant and dissonant musical intervals (Bidelman and Krishnan 2009). Our methods were sufficient for observing the FFR, but insufficient for detailed FFR analysis (Skoe and Kraus 2010). However, the frequency ratios of *Xenopus* calls and the dyads used as test stimuli fit the intervals of the

Western musical scale, so we determined whether the consonance or dissonance of a frequency pair contributed to AEP threshold, or P1 peak amplitude and latency at 100 dB. Dyads were ranked in order of consonance based on human psychophysical reporting (Malmberg 1918; Bidelman 2013). There were no correlations between consonance and threshold, peak latency or amplitude either for dyads that contained one common frequency or across all dyads (Pearson's R, all $p > 0.05$, data not shown).

Frog species, sex and size influenced AEPs

Frogs can exhibit size-related differences in auditory sensitivity and behavioral responses to sound (Zakon and Wilczynski 1988; Keddy-Hector et al. 1992; Fox 1995; Meenderink and Narins 2010), and across our population sex and species were the greatest contributors to size variation. To investigate whether size, sex, and species contribute to variation in AEP signal amplitude, we used these variables in a linear model predicting the highest amplitude response measured, the P1 in AEPs to clicks presented at 111 dB SPL. Across all the frogs tested, frog mass was the best single predictor of the P1 peak amplitude (Fig. 8, Regression $F_{(1,102)} = 28.32$, $p < 0.0001$, $R^2 = 0.2173$) with a negative correlation between mass and P1 peak amplitude.

Incorporating sex as an indicator variable (Male? [0 = No, 1 = Yes]) yielded a negative coefficient and significantly improved the model (Forward selection, $F_{(2,101)} = 20.80$, $p < 0.0001$, $R^2 = 0.2917$), suggesting that males had smaller AEP amplitudes than females. Males were smaller than females in all species (mass and snout-vent length, t-tests, all species $p < 0.001$), so the difference between the sexes ran counter to the general observation that smaller frogs have larger AEP peaks. Frog mass co-varied with species; the largest frogs were always *X. laevis* and the smallest were *X. amieti*, but *X. borealis* and *X. petersii* were similar in body mass. To disambiguate the role of species and size, we added a categorical variable for *X. petersii* which significantly improved the model (Forward selection, $F_{(3,100)} = 19.36$, $p < 0.0001$, $R^2 = 0.3675$), suggesting that AEP amplitude differed between species, even those with similar mass. Indeed, a model consisting of only categorical variables for species was significant ($F_{(3,100)} = 19.17$, $p < 0.0001$, $R^2 = 0.3651$).

To clarify the statistical relationships between AEP amplitude and species, sex, and mass observed in the full model that grouped all species, we examined the relationships between AEP amplitude, sex, and mass within each species. Within species, there were no significant correlations between AEP amplitude and frog mass or snout-vent length (Pearson's R, all $p > 0.05$). As in the full model, males tended to produce smaller AEP peaks than females, but this was only significant for *X. amieti* and *X. petersii* (Student's t, $p = 0.050$ and 0.009 , respectively). There was no sex difference in signal amplitude in *X. borealis* and *X. laevis*, demonstrating that the sex difference in AEP signal may be species-dependent. Between the two species of similar size, *X. borealis* and *X. petersii* (mass, snout-vent length; Student's t, both $p > 0.41$), there was a significant difference in AEP signal amplitude (Student's t, $p = 0.001$). Therefore, since sex and mass were not strong predictors of AEP amplitude within species, species identity appeared to be the major contributing factor to the relationships observed between sex, mass, and AEP amplitude.

Anesthetic Comparison

Anesthesia and anesthetic state greatly influence the quality of auditory recordings (Katbamna et al. 2006b; Schumacher et al. 2011). To examine the effects of anesthetic state on AEP quality, we compared AEPs from frogs immobilized with d-tubocurarine to those anesthetized with tricane methanesulfonate (MS222), a commonly used frog and fish anesthetic. Frogs were immersed in 1% MS222 until deeply anesthetized, determined by lack of response to toe pinch and loss of righting reflex. Exposure to MS222 until the frogs were fully anesthetized resulted in a small AEP that continued to decrease over the first 30–45 m of recording.

To obtain larger, more stable AEPs with MS222, we developed a two-step anesthesia protocol: immersion in 1% MS222 solution for 3 – 7 m, depending on body size, and then transferring the awake frog back to its home tank until the anesthetic had time to take complete effect (5 – 7 m). If ineffective, the frog was re-immersed in MS222 for 30 – 60 s followed by 5 m in home water. This protocol resulted in shallow anesthesia; there was no righting response, nor response to toe pinch but frogs recovered more rapidly. When necessary, supplemental anesthesia was administered during recording sessions as 5 – 10 drops of 1% MS222 onto the Kimwipe draped over the frog; recording resumed 5 – 10 m later.

Frogs immobilized with curare produced AEPs of greater amplitude that were more stable over time than frogs anesthetized with MS222 (Fig. 9A). Even when the minimum amount of MS222 required to anesthetize a frog for the recording session was delivered, AEP amplitudes to clicks were 63% smaller than those recorded under curare (*X. petersii* females, MS222 n = 8, curare n = 14; Student's T, p = 0.01). The effects of MS-222 increased with time (Fig. 9a). For example, in *X. petersii* females anesthetized with MS222, AEP amplitudes decreased significantly from baseline recordings within an hour (Repeated Measures ANOVA, $F_{(2,7)} = 9.083$, p = 0.003, Dunnett's post hoc p < 0.05). When immobilized with curare, the amplitude of the AEP to a 111 dB click did not change over the course of the experiment. Decreases in spectral sensitivity were also observed under MS222; the audiograms of MS222-treated *X. amieti* females (n = 11) did not display regions of enhanced sensitivity seen in audiograms recorded from curarized *X. amieti* females (n = 14; Fig. 9b).

Decreases in signal quality were also observed when MS222 anesthesia was administered acutely. In the example of Fig. 9c, a curarized *X. laevis* female was given a 1.5 mL injection of 1% MS222 into the dorsal lymph sac. Over the next 22 minutes, the AEP amplitude to a 111 dB click decreased by 74%. Because of the effects of MS222 on signal quality, all of the results presented outside of this section are from frogs treated only with curare.

Discussion

Our results reveal that *Xenopus* have sexually dimorphic, endocrine-mediated frequency tuning. Female *Xenopus* in three of the four species examined were especially sensitive to the spectral features of conspecific male calls. In female *X. laevis*, the sensitivity to these

spectral features was modulated by ovarian signals. We examined factors that could influence AEPs and compared *Xenopus* AEPs to those of other species. We also discuss potential mechanisms for and the ecological significance of dyad sensitivity.

AEP quality

The AEPs obtained from *Xenopus* share characteristics with AEPs from other animals, including humans (Corwin et al. 1982; Britton-Powell et al. 2002; Katbamna et al. 2006a; Katbamna et al. 2006b; Hall 2007; Schrode et al. 2014; Vélez et al. 2015). The AEPs demonstrate level dependence; amplitude increases and latency decreases with stimulus intensity. We also observed variations in AEP waveform shape correlated with stimuli. These shape differences were related to differences in threshold to the stimuli presented and the envelope modulation of dyads.

Species identity appeared to make the greatest contribution to AEP amplitude. Larger frogs might be expected to have larger auditory organs and brains yielding higher amplitude AEPs. However, a 4 g *X. amieti* produces a larger AEP than a 150 g *X. laevis*. The relationship between body size and AEP amplitude is inverse and appears only across rather than within species. Skull thickness and the size of the ear or auditory nerve contribute to AEP amplitude (Mitchell et al. 1989). The relative position of the recording electrodes was kept constant for all species, but the absolute distance from the AEP source was greater in species with larger skulls and more surrounding tissue. Within each species, females are larger in body size but, at least in *X. laevis*, the absolute area of the tympanic disk is similar (Mason et al. 2009). Relative to body size, the tympanic disk of females is smaller than that of males (Mason et al. 2009).

These experiments were conducted at ambient temperature, between 22.5 and 24 °C. Changes in neural and hair cell activity has been observed in isolated preparations, even within this narrow temperature range (Smotherman and Narins 1998). How this temperature range influenced our *in vivo* recordings is not clear but a previous study in *Xenopus* reported no effect of variations in temperature between 17 and 20 °C on VIIIth nerve and dorsal medullary nucleus activity (Elliott et al. 2007).

One particularly noteworthy finding for future anuran AEP studies is the detrimental impact of MS222 anesthesia. MS222 is widely used in anuran AEP experiments, but anesthesia can diminish auditory sensitivity and recording quality (Katbamna et al. 2006b; Schumacher et al. 2011). Our recordings clearly demonstrate that MS222 anesthesia decreases overall auditory sensitivity and that alternative methods of immobilization should be used in auditory recordings. One potential alternative is benzocaine, which does not appear to have the same detrimental effects on hair cell activity as MS222 (Balak et al. 1990; Meyers et al. 2003) but is also associated with more health risks and mortality (Cakir and Strauch 2005; Cacala et al. 2007). In contrast to curare, a recent paper reports that both benzocaine and MS-222 block sensory nerve activity in *Xenopus* (Ramlochansingh et al. 2014), again supporting the use of alternative means of immobilization for recordings of neural activity.

Dyad sensitivity

The salience of acoustic features in courtship vocalizations might differ for males and females. The advertisement call of male *Xenopus* has a spectrally distinctive acoustic feature, a frequency dyad. Female *Xenopus* are especially sensitive to the dyads that make up their species' male advertisement call while males tended to be most sensitive to the lower frequencies of male-directed calls.

Small shifts of either DF1 or DF2 away from the SOD abolished the auditory advantage of dyads for females. Females are attracted to male advertisement calls (Picker 1983) and their heightened, peripheral acoustic sensitivity to the SOD could contribute to locating a distant, calling male or to detecting the call when ambient noise levels are high, as would be predicted by the matched filter hypothesis (Frishkopf et al. 1968; Capranica and Moffat 1983; Moreno-Gomez et al. 2013; Simmons 2013). Male advertisement calls are also socially salient for other males; in *X. laevis* they support vocal dominance and suppress calling in rival males (Tobias et al. 2004). In males, enhanced sensitivity to distant calling males could be disadvantageous by enhancing vocal suppression a factor that might explain why we found that males were not peripherally tuned to the spectral features of the SOD.

Though matched auditory filters in anurans have been studied previously, sex differences in frequency tuning have been reported in only a few species (Narins and Capranica 1976; Wilczynski et al. 1984; Wilczynski et al. 1992; Gerhardt and Schwartz 2001). Seasonal variation associated with the reproductive cycle and the effect of social signals on the auditory system suggest that gonadal hormones influence tuning (Hillery 1984; Penna et al. 1992; Goense and Feng 2005; Arch and Narins 2009; Gall and Wilczynski 2015). The differences between intact and OVX female *X. laevis* described here support this idea. While there is ample evidence for androgen synthesis within the brains of male and female frogs (Mensah-Nyagan et al. 1996; Bruzzone et al. 2010; Vaudry et al. 2011), in *Xenopus* our results suggest instead that circulating ovarian hormones generate the female auditory matched filter.

Tuning differences between intact, OVX and OVX+DHT *X. laevis* demonstrate plasticity in the adult peripheral auditory system and suggest hormones modulate sensitivity to species-specific DFs. The onset latency of the threshold responses (3 – 5 ms) suggests that they reflect auditory nerve activity. Thus, loci for androgen action could be the hair cells in the auditory periphery (Forlano et al. 2015), or neurons in the acoustic ganglion expressing androgen receptor (Perez et al. 1996). Androgens contribute to feminization or have female-specific effects in other species and are the primary steroid produced in the *Xenopus* ovary (Lutz et al. 2001). In zebra finches, which also have sexually dimorphic hearing (Phan et al. 2006; Yoder et al. 2015), estradiol has been implicated in the acute and chronic modulation of the auditory system (Remage-Healey et al. 2012; Yoder and Vicario 2012). Though DHT is not aromatized to estradiol, conversion to 5 α -androstane-3 β ,17 β -diol might permit interaction with estrogen receptors in the inner ear (Forlano et al. 2015). Estradiol and other ovarian signals absent in males could be necessary for the enhanced auditory sensitivity of females.

In this study we found no influence of sex or endocrine state on the FFR and only a minimal influence on the click-evoked P1 amplitude. Click stimuli contain frequencies that stimulate both the amphibian and basilar papillae. The results of the masking study suggest that the FFR was generated by the amphibian papilla, but the higher frequencies of advertisement calls should instead excite the basilar papilla. Thus, the basilar papilla is a candidate site of plasticity contributing to heightened auditory sensitivity in females.

The FFR in communication

The FFR, when measured with AEP, reflects sustained neural activity that is phase-locked to individual cycles of the stimulus waveform or modulations of the stimulus envelope.

Bullfrogs (*Bufo americanus*), leopard frogs (*Rana pipiens*), and green tree frogs (*Hyla cinerea*) are sensitive to envelope modulations and harmonic structure, as demonstrated by behavioral (Gerhardt 1978; Simmons 1988; Hainfeld et al. 1996; Simmons and Bean 2000) and electrophysiological (Capranica and Moffet 1980; Corwin et al. 1982; Klump et al. 2004; Goense and Feng 2005) evidence. While our 10 ms recording window and stimulus duration were shorter than those typically used for recording FFRs, our AEP waveforms show clear correlations between their peak frequency and the beat frequency of the stimulus dyad (Fig. 4) that are level dependent (Fig. 3, Worden and Marsh 1968) and are consistent with the FFR range recorded in other frogs (Corwin et al. 1982; Dunia and Narins 1989; Freedman et al. 1989; Narins and Wagner 1989). Moreover, the 10 ms stimuli we used to evoke the FFRs are similar in length to the sound pulses used in *Xenopus* communication vocalizations (Vignal and Kelley 2007) and therefore a more biologically relevant stimulus for these species.

By using a band-limited masker, we provide evidence that the FFR was generated by the amphibian papilla. The amphibian papilla contains 250–1200 hair cells, is tonotopically organized, and sensitive to frequencies from 100 – 1000 Hz, depending on species (Lewis et al. 1982; Fox 1995; van Dijk et al. 2011). In contrast, the basilar papilla contains 20 – 200 hair cells, typically has higher thresholds than the amphibian papilla, and is sensitive to frequencies from 1 – 4 kHz, depending on the species (Lewis et al. 1982; Fox 1995; Serrano et al. 2001; van Dijk et al. 2011). The basilar papilla is not tonotopically organized but its cells are still frequency sensitive (Wilczynski et al. 2001; van Dijk et al. 2011). By continuously masking the activity of the amphibian papilla and degrading the FFR, we demonstrated that it was contributing to dyad-evoked AEP waveforms. However, the invariance of the FFR across species, sex, and endocrine state suggests that beat frequencies do not underlie the female sensitivity to their SOD.

All four species and both sexes showed similar FFR patterns, suggesting that the FFR may not be specialized for species-specific communication and does not contribute to sex differences in dyad sensitivity. The call types of *X. laevis* (trill), *X. petersii* (trill), *X. amieti* (burst), and *X. borealis* (click) vary greatly in their temporal qualities but FFRs up to 700 Hz would not be a limiting factor in processing the temporal qualities of vocalizations; the fastest pulse rate (~140 Hz) is seen in the bursting call of *X. amieti* (Tobias et al. 2011). However, the beat frequency of *X. petersii* dyads is near peak frequency, latency, and

amplitude optima of the FFR and could facilitate the peripheral processing of vocalizations in this species (Rose and Capranica 1985).

In humans, the FFR is correlated with the perception of speech sounds and music (Krishnan et al. 2010; Bones et al. 2014), and can be influenced by experience, particularly musical training (Chandrasekaran and Kraus 2010; Lerud et al. 2014). The frequency ratios of dyad stimuli in *Xenopus* advertisement calls form intervals in the Western musical scale. However, we found no correlation between our AEPs and the consonance or dissonance of the dyad intervals. This finding does not preclude the possibility of frequency ratio sensitivity at higher levels in the auditory system (Bidelman and Krishnan 2009; Skoe and Kraus 2010).

Though the FFR to beat frequencies showed no species-specific specializations, it may contribute to the neural coding of sound localization. The interference with the FFR produced by a 100–700 Hz band limited masker suggests that auditory nerve fibers contributing to the FFR respond to the frequency difference $F2 - F1$, as has been observed in bullfrog (Capranica and Moffatt 1980). At low frequencies the envelope periodicity encoded by these neurons could facilitate sound source localization by interaural time differences (Capranica and Moffatt 1980; Klump et al. 2004). This mechanism could contribute to dyad-mediated mate localization during phonotaxis.

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ABBREVIATIONS

ABR	auditory brainstem response
AEP	auditory evoked potential
ANOVA	analysis of variance
DF	dominant frequency
DHT	dihydrotestosterone
F1	frequency one
F2	frequency two
FFR	frequency following response
MS222	tricane methanesulfonate
N1	first negative AEP peak

OVX	ovariectomized
OVX+DHT	ovariectomized and given DHT
P1	first positive AEP peak
P2	second positive AEP peak
SOD	species own dyad
TDT	Tucker Davis Technologies

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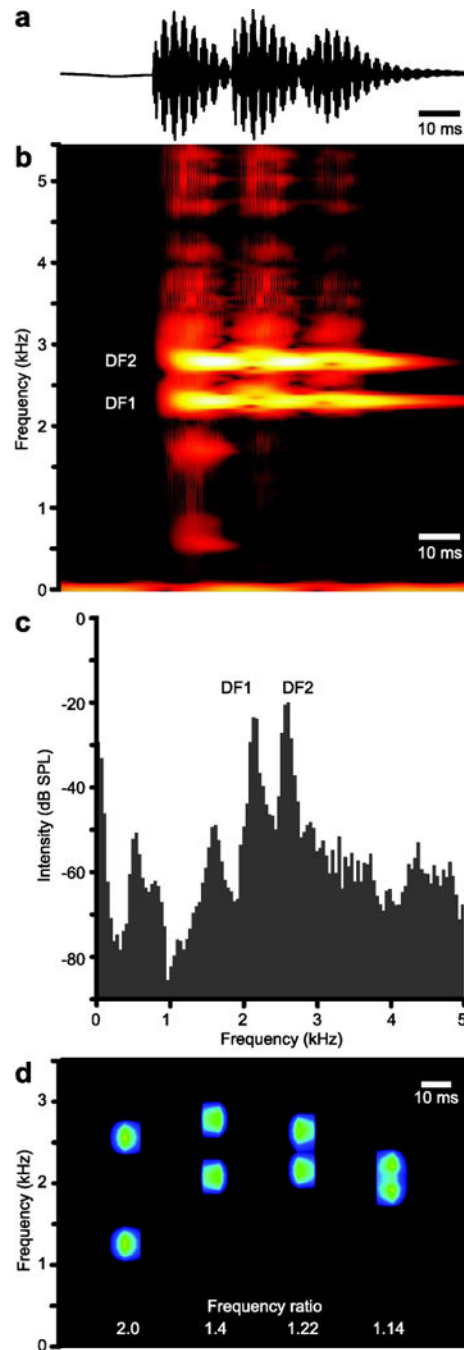


Fig. 1.

a) An oscillogram of three sound pulses from a male *X. petersii* call. The spectrogram **(b)** and frequency power spectrum **(c)** of the call in **(a)** illustrates the two, distinct frequencies of high intensity within each pulse. The lower frequency is DF1, the higher DF2. **d)** A spectrogram of the synthetic SODs for *X. borealis*, *X. amieti*, *X. petersii*, and *X. amieti* (left to right) with the frequency ratio identified below each sound pulse. In this spectrogram,

intensity is coded in blue-green instead of red-yellow (as in b) to distinguish the *in vitro* and *in vivo* sound sources.

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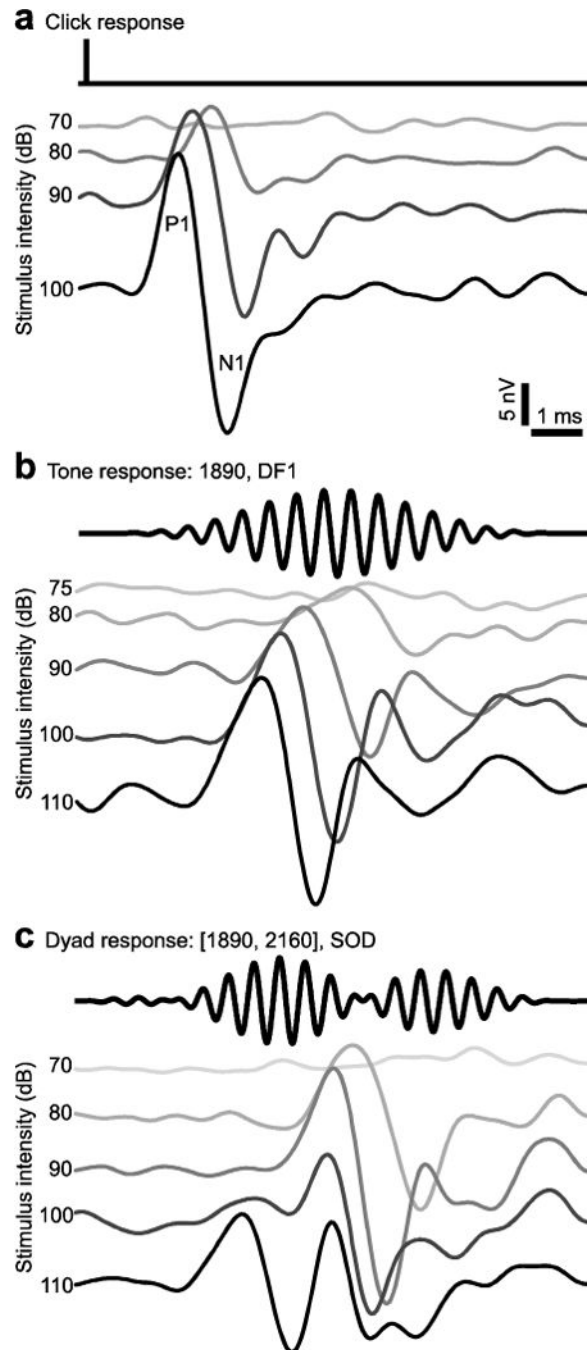


Fig. 2. AEPs from a single *X. laevis* female (DF1: 1890, DF2: 2160, Frequency ratio: 1.14) illustrate the differences between responses to clicks (**a**), tones (**b**), and dyads (**c**). Oscillograms of stimuli are in black above each waterfall plot, stimulus intensities are to the left of each AEP. The first positive (P1) and negative (N1) peaks are indicated in (a).

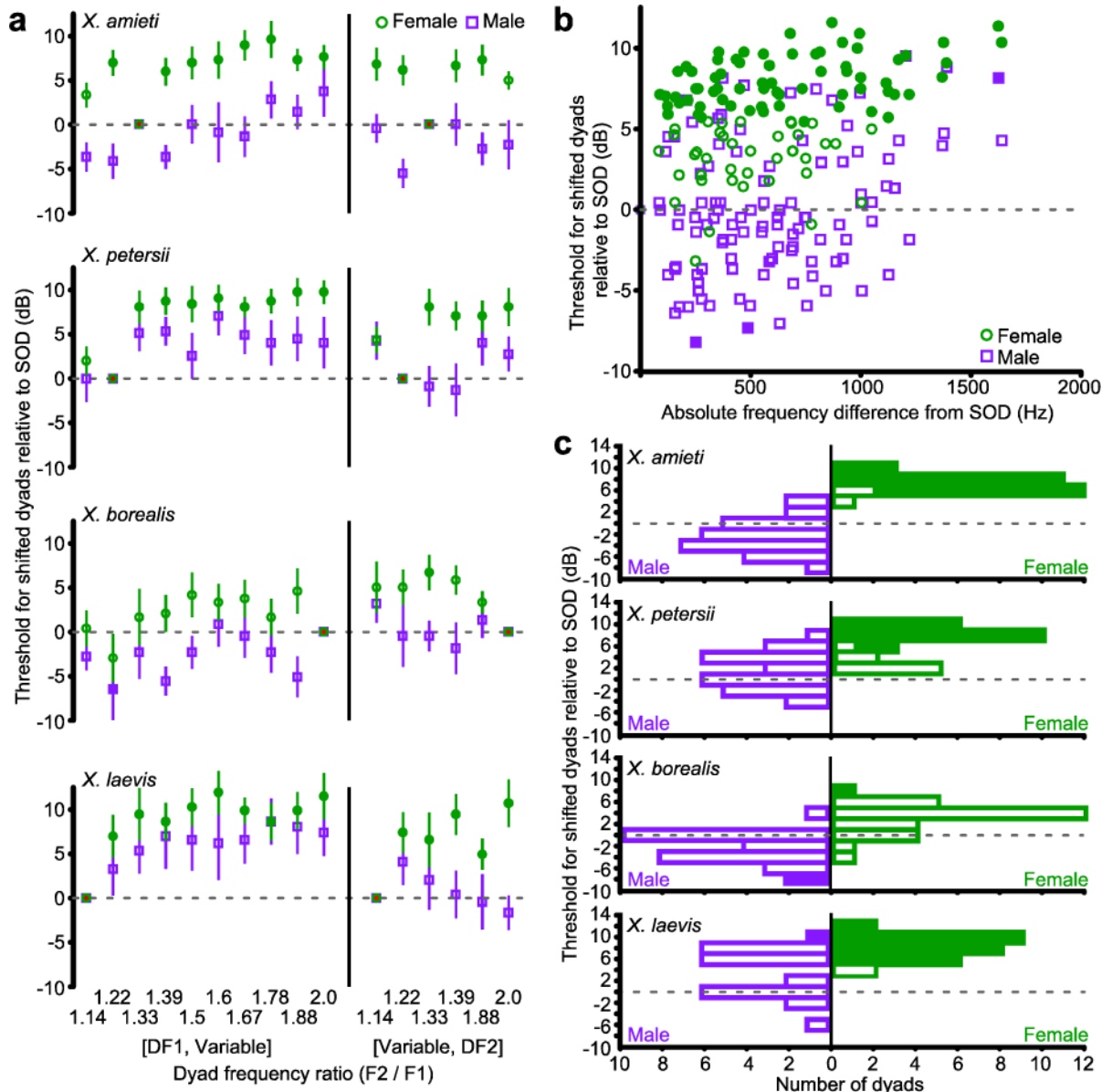


Fig. 3.

a) AEP thresholds for dyads containing at least one DF from the SOD, in females (green circles) and males (purple squares). Filled symbols indicate thresholds that differ significantly from the SOD threshold (Repeated Measures ANOVAs with Dunnett's multiple comparisons post hoc, all $p < 0.05$). The dashed line indicates the threshold for the SOD. Error bars indicate standard error. **b)** The absolute frequency difference from SOD was calculated as the sum of the absolute differences between each DF and the closest frequency in the altered dyad. All dyads (not just the DF1 or DF2 manipulations shown in (a)) are illustrated and species are grouped using the color conventions in (a). **c)** The data in (b) presented as a histogram of thresholds for all dyads relative to the threshold for SOD by species. For female *Xenopus* (green), thresholds for most other dyads are significantly

higher (Filled blocks represent dyads with thresholds significantly different from the SOD (dashed line).

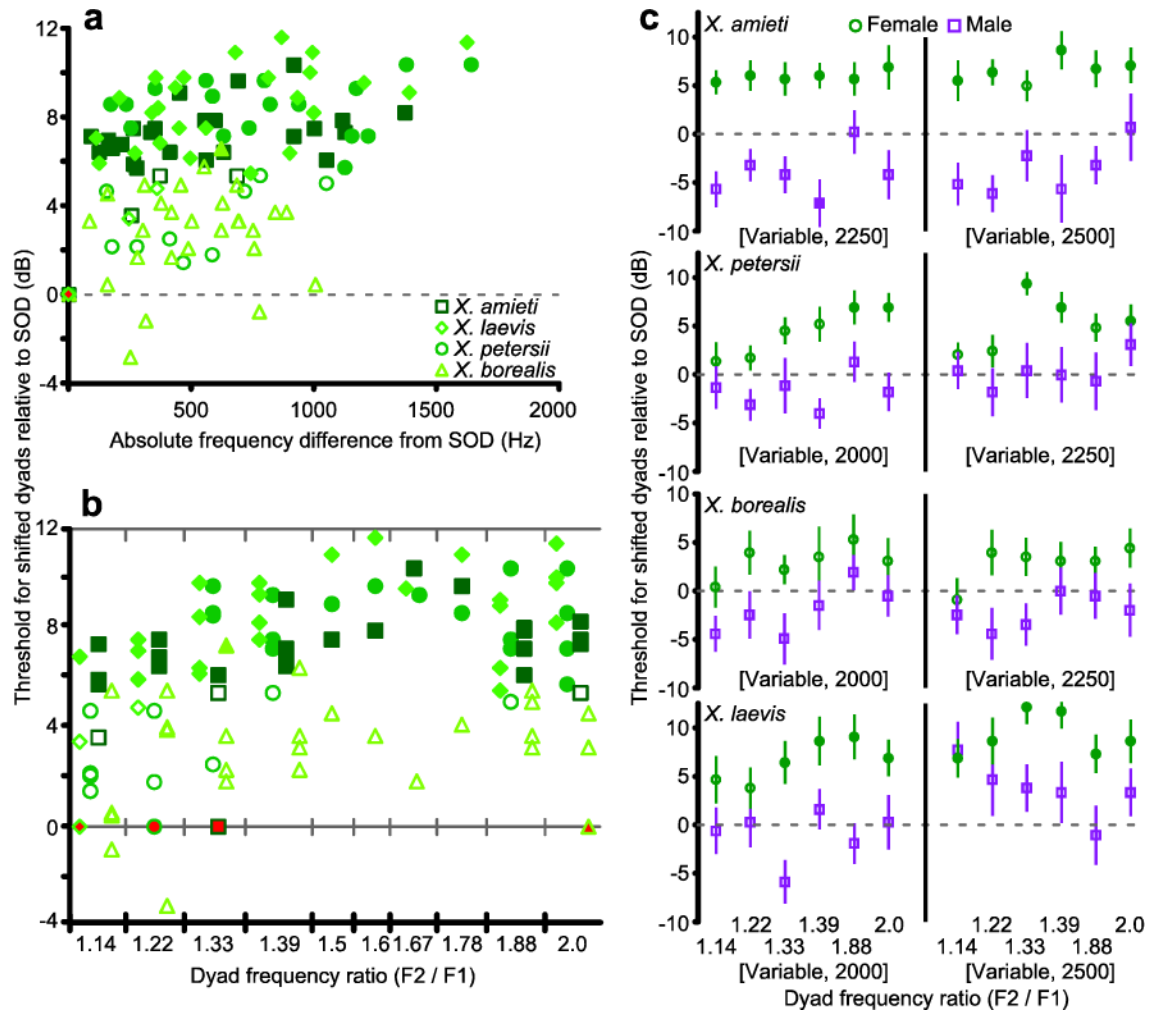


Fig. 4.

a) Female data from Fig. 3b separated by species: *X. amieti* (squares), *X. laevis* (diamonds), *X. petersii* (circles), and *X. borealis* (triangles). As in Fig. 3b, filled symbols indicate significantly higher thresholds than the SOD threshold (determined by the same Repeated Measures ANOVA in Fig. 3). **b)** The dyads in (a) separated by frequency ratio. Dyads with AEP thresholds that differed significantly (same Repeated Measures ANOVA in Fig. 3) from the SOD (filled in red) are filled in green. **c)** AEP thresholds for dyads composed of arbitrary frequencies that did not contain DF1 or DF2. Filled symbols indicate thresholds that were significantly different (determined by the same Repeated Measures ANOVA in Fig. 3) from the SOD (dashed line). Error bars indicate standard error.

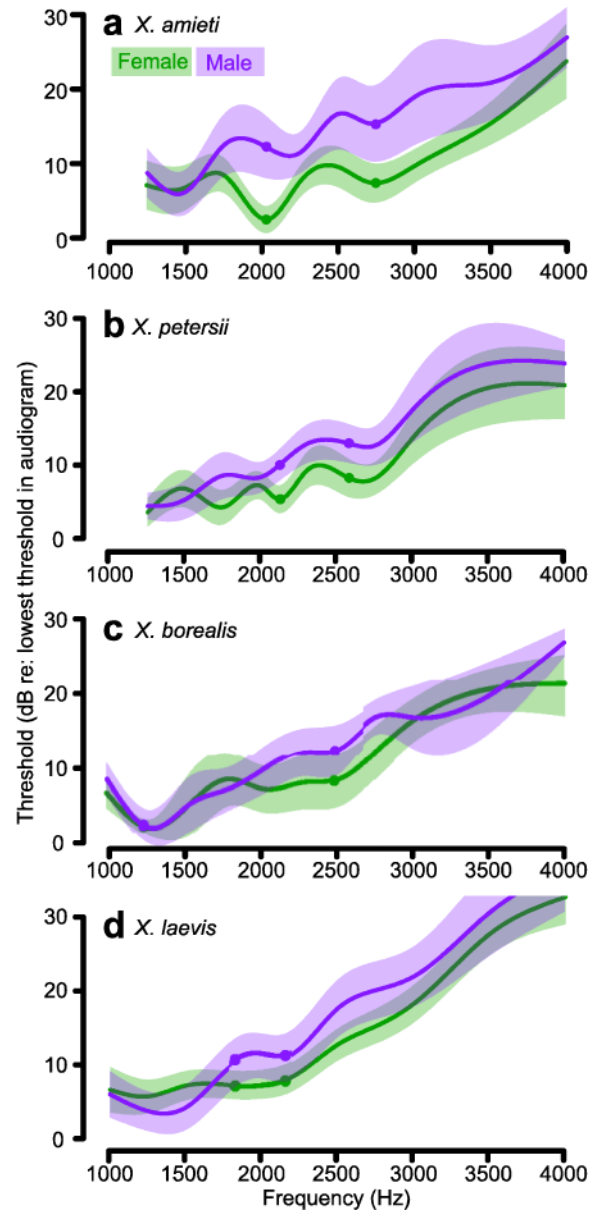


Fig. 5.
a–d) Female (green) and male (purple) audiograms from four species of *Xenopus*. Average values and 95% confidence intervals (shaded regions) for each sex and species are shown. Before averaging, individual audiograms were normalized to the frequency of lowest threshold to facilitate comparison of audiogram shape. DF1 and DF2 of the SOD are indicated by filled circles.

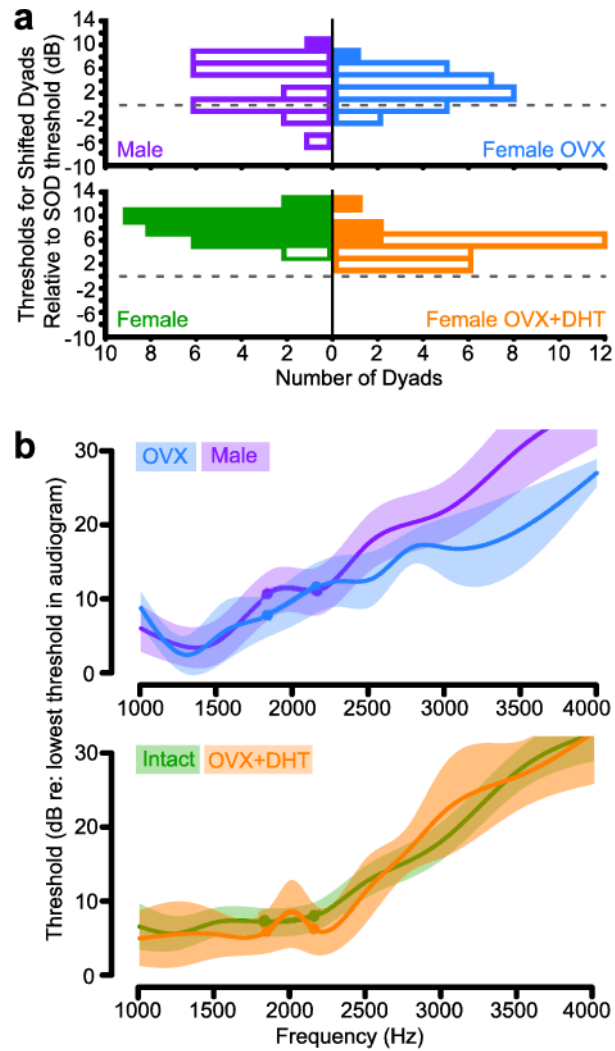


Fig. 6.

a) Using the conventions in Fig 4a, intact female (green) and male (purple) *X. laevis* are compared to ovariectomized *X. laevis* females (OVX, blue) and ovariectomized *X. laevis* females treated with DHT (OVX+DHT, orange). **b)** Using conventions from Fig 5, the average OVX audiogram (blue) is compared to that of males (purple) and the average OVX +DHT audiogram (orange) is compared to that of intact females (green).

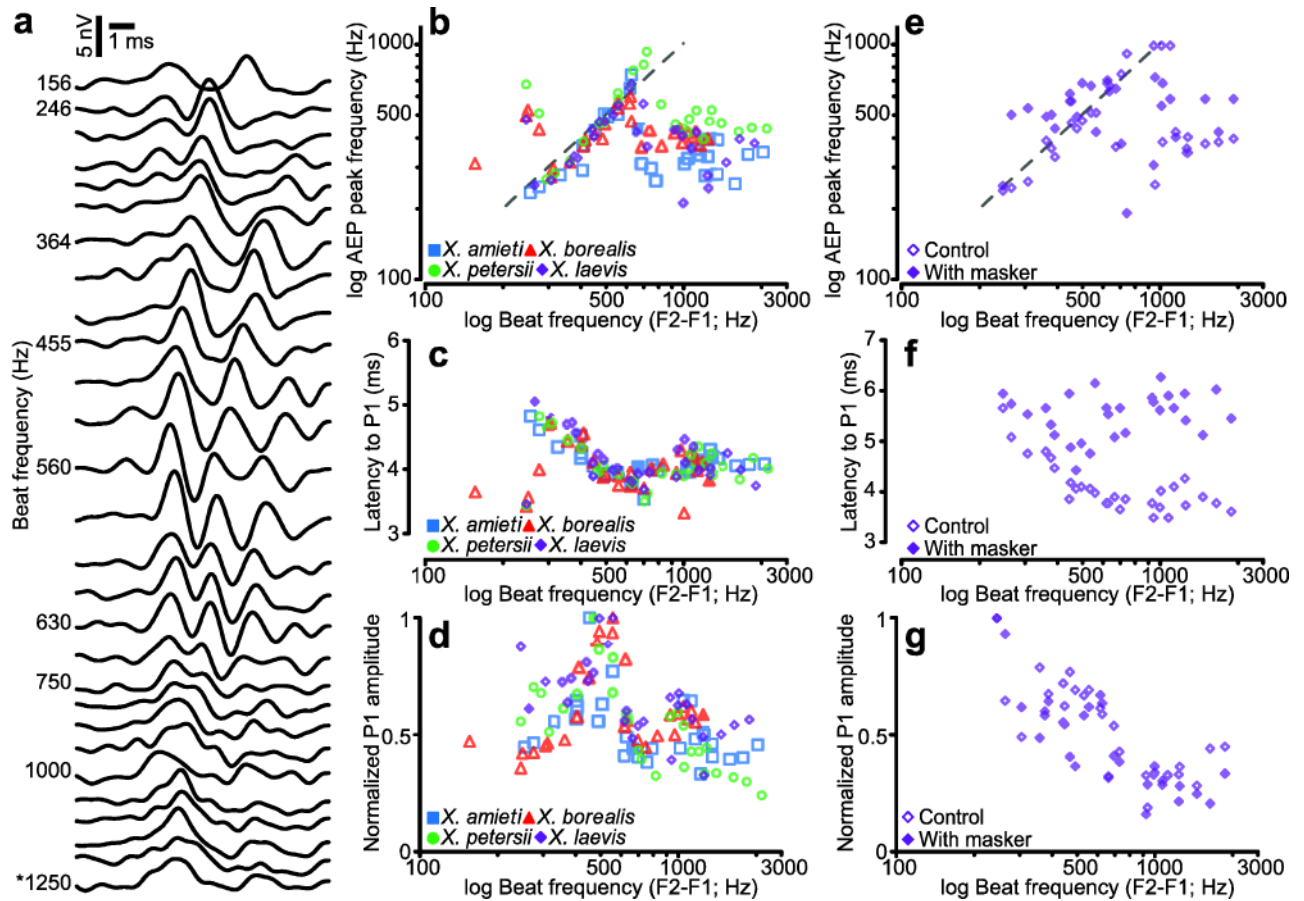


Fig. 7.

AEP waveforms varied with the beat frequency of dyads. **a)** A waterfall plot of averaged female *X. borealis* AEPs with increasing beat frequency (F2–F1) towards the bottom. The asterisk indicates the AEP to the SOD. **b)** The correlation between the frequency of AEP peaks (P1, P2) and the beat frequency of the dyad from 200–700 Hz. The dashed line is $y = x$. The correlation between beat frequency and P1 latency (**c**) and P1 amplitude (**d**) are also plotted. The SOD for each species is indicated by the filled symbol. **e, f, and g** plot AEP peak frequency, P1 latency, and amplitude, respectively, using the same conventions as **b, c, and d**. This subset of animals was exposed to dyads alone (open symbols) and in the presence of a 100–800 Hz masker (filled symbols).

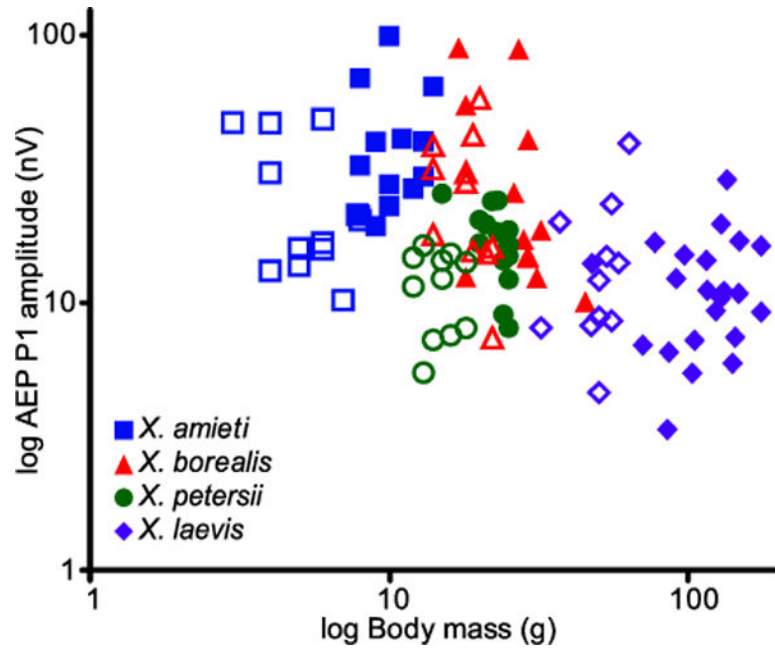


Fig. 8. The relationships between AEP amplitude (P1 amplitude of 111 dB click response) and body mass, species (color and shape), and sex (males open, females solid) are plotted.

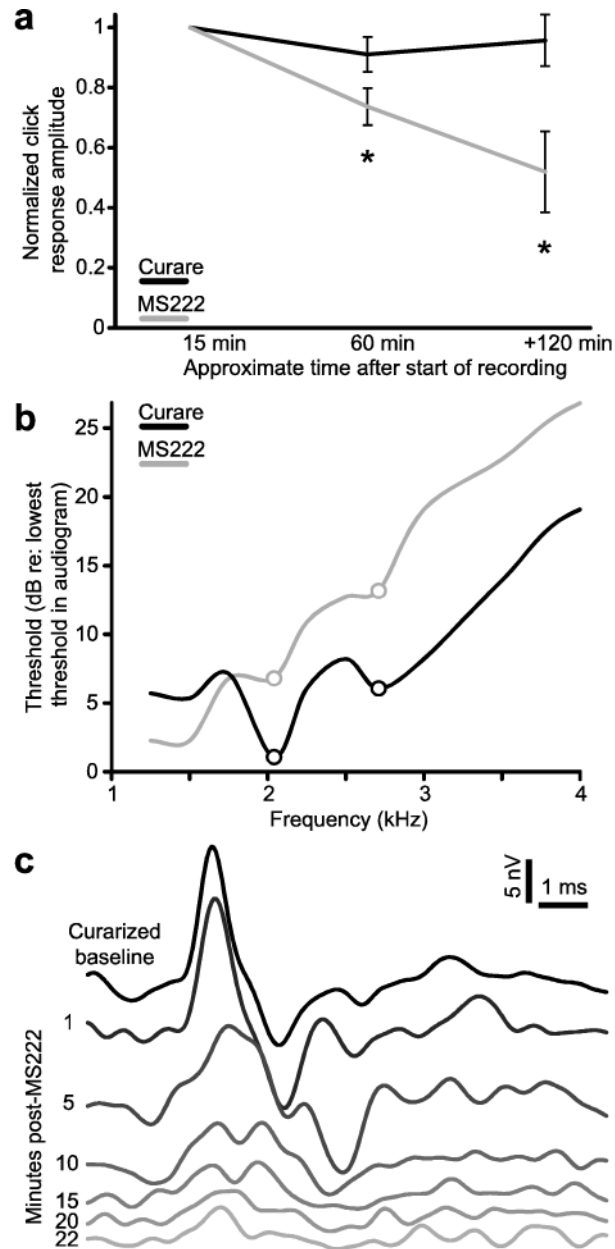


Fig. 9.

a) The P1 amplitude of AEPs to clicks in frogs treated with MS222 (gray; $n = 8$ *X. petersii* females) and curare (black; $n = 14$ *X. petersii* females). **b)** Average audiograms recorded under MS222 anesthesia (gray; $n = 11$ *X. amieti*) and when curarized (black, $n = 14$ *X. amieti* females). DF1 and DF2 are indicated by circles. **c)** The AEP of a curarized *X. laevis* female to a 111 dB click stimulus before and after injection of an anesthetizing dose of MS222 into the dorsal lymph sac.