

Early Experience Leads to Changes in the Advertisement Calls of Male *Physalaemus pustulosus*

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Anurans have long been a model system for studies of animal communication, but little is known about how individuals acquire acoustically linked mating behaviors. The manner in which behaviors are acquired may be a source of variation in these behaviors. In this study, we reared *Physalaemus pustulosus* in four acoustic treatment groups: hearing a conspecific chorus of *P. pustulosus* frogs, acoustically isolated from all frog calls, hearing a chorus of the congener *P. enesefae*, and hearing noise. We then measured the spectral and temporal characteristics of the calls produced by males and tested them for differences between treatment groups. Males reared in isolation produced calls that were shorter in duration. Females showed discrimination against these shorter calls. Early experience can alter the advertisement call of male *P. pustulosus*, and acoustic isolation leads to the acquisition of calls that are less attractive to females. Males reared in other groups produced species-typical calls. The calls of male *P. pustulosus* may be the product of a gene by environment interaction.

MATING behaviors can be acquired by learning, determined by genetics, or the product of a gene by environment interaction. Determining which of these mechanisms is involved in the acquisition of a species' mating behaviors is critical to understanding sources of variation in those behaviors.

Acquisition of acoustic signals by vocal learning during early social interactions has been extensively studied in songbirds. Observations of dialects in populations of white-crowned sparrows in central and northern California (Marler and Tamura, 1962) led to studies of lab-reared sparrows, which showed that they acquire their songs by cultural transmission (Marler and Tamura, 1964). Atypical songs are produced when males are reared in isolation (Thorpe, 1958) or with heterospecific tutors (Marler and Tamura, 1964). Divergence in birdsong may be a reproductive isolating mechanism in speciation (Seddon and Tobias, 2007).

Another classic model for studies in animal communication is the acoustic behavior of anurans (reviewed in Ryan, 2001; Gerhardt and Huber, 2002). Advertisement calls produced by male frogs and toads have shown to be a potential reproductive isolating mechanism (Blair, 1941). This was reinforced when it was demonstrated that females are attracted to conspecific calls (Martof and Thompson, 1958) and that they can discriminate between closely related species solely on the basis of advertisement calls (Littlejohn and Michaud, 1959).

One of the original researchers in birdsong commented that a wide range of taxa—from insects to amphibians—have elaborate vocalizations that might be of interest in studies of vocal learning (Thorpe, 1961). Geographic variation in mating calls has been documented in many anurans (Capranica et al., 1973; Nevo and Capranica, 1985; Asquith et al., 1988; Ryan and Wilczynski, 1991; Ryan et al., 1996); however, in no case was there evidence for the sharp boundaries that are associated with songbird dialects. The lack of dialects, however, does not preclude the possibility that early experience influences anuran mating behaviors.

Given the importance of communication in anuran reproductive behavior, it is surprising that there are no published studies about the role of early experience in the

acquisition of anuran acoustic behaviors. These behaviors could be acquired through genetic determination, showing little variation with experience and developmental environment, or they could be the sole product of experience. An intermediate possibility is that the behaviors are the result of the interaction of genes and environment, with experience modulating the expression of genetically-based pathways. Although no studies of anurans have specifically addressed the issue of how males acquire their calls, observations of naturally-occurring hybrids suggests a genetic basis for this behavior (Gerhardt et al., 1980).

In a well-studied neotropical frog, *Physalaemus pustulosus*, males produce either a simple (whine) or complex (whine-chuck) advertisement call (Fig. 1A). The whine is an amplitude-modulated, downward frequency-sweep. The escalation from simple to complex calls is facultative, and females prefer the complex call (Ryan, 1980; Rand and Ryan, 1981). Males typically choose to escalate to the complex call only when other nearby males are calling (Ryan, 1985). Frog-eating bats preferentially prey on males making the complex call (Ryan et al., 1982; Tuttle et al., 1982).

The genus *Physalaemus* is widespread throughout South America, with *P. pustulosus* being the sole member of the species in Middle America. *Physalaemus pustulosus* is allopatric with most species with the exception of *P. enesefae*, which is sympatric in the llanos of Venezuela (La Marca, 1992). Male *P. enesefae* produce only simple calls, with a similar downward frequency sweep, but their calls are longer in duration compared to the calls of *P. pustulosus* (Fig. 1B). To avoid the high cost of breeding with heterospecifics, females in the area of sympatry must discriminate against the calls of *P. enesefae* and they do so in a probabilistic fashion in which internal and external factors contribute to the probability that a given female will respond to a given signal (Ryan et al., 2003). In addition, female *P. pustulosus* discriminate against a number of synthetic calls that are intermediate between the conspecific call and the call of *P. enesefae*. Their results show the point where female response reaches a specific level, so those synthetic calls can be used to test thresholds for female preferences.

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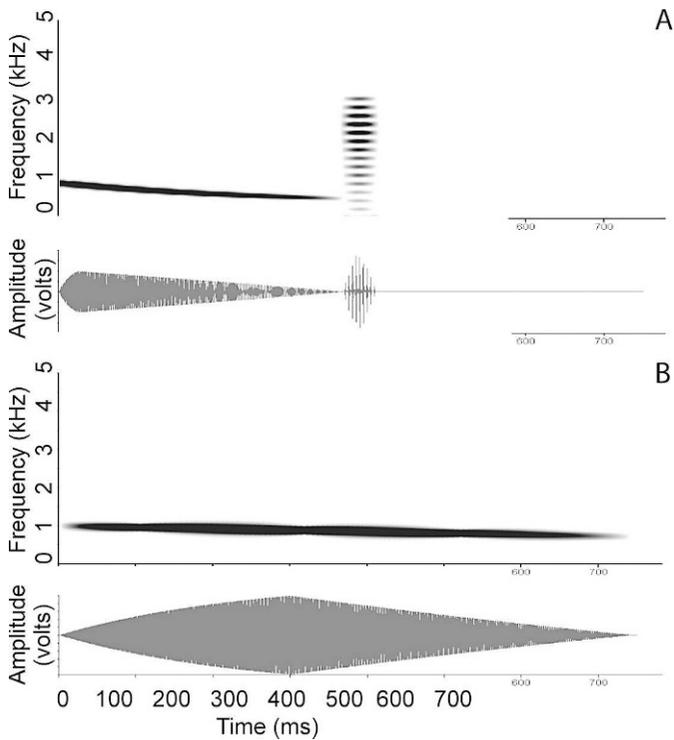


Fig 1. Typical advertisement call of male *Physalaemus pustulosus* (A) and *Physalaemus enesefae* (B). Male *P. pustulosus* produce an amplitude modulated call that rises to maximum amplitude quickly. When other males are calling, male *P. pustulosus* often add one or more “chucks” at the end of the call. Male *P. enesefae* produce a similar call, but the rise to maximum amplitude and overall call duration are longer.

In this study, we use the classic experimental design used in songbird research to investigate the possible contribution of early experience to the development of advertisement calls in male *P. pustulosus*. Acoustic rearing conditions provide a range of early experiences against which we test the effects of this experience on the advertisement call of male Túngara Frogs.

MATERIALS AND METHODS

Male calls.—In a laboratory facility at the University of Texas at Austin, 300 *P. pustulosus* from 20 broods were reared from tadpoles through sexual maturity in sound-attenuated enclosures approximately 32 cm wide, 60 cm long, and 18 cm tall. Each brood was separated into one of four rearing conditions: chorus of calls of *P. pustulosus*, isolated from all frog calls, chorus of calls of *P. enesefae*, and noise. The playback for the groups hearing anuran choruses were broadcast at 80 dB SPL, approximately the amplitude of a calling male measured at one meter, and played for 12 hours during each night photoperiod. The noise was also broadcast at 80 dB SPL, but played for 24 hours to simulate noise exposure from an abiotic source such as a waterfall or an anthropogenic source such as a highway. All animals were fed *ad libitum* and received 12 hours of broad-spectrum light daily. No standing water was present in the enclosures; this species needs water to call, so the lack of standing water prevented males in the enclosures from calling. There were four replicates of each rearing group, for a total of 16 enclosures. All frogs were maintained in these acoustic

environments for eight to ten months, until they reached sexual maturity (24 mm snout–vent length).

At sexual maturity, each male was placed in an individual sound-attenuated chamber (22 cm wide, 24 cm long, 40 cm tall) and presented with a small pond of water from which to call. These chambers were constructed of 2.54 cm thick medium density fiberboard and lined with 2.54 cm thick acoustic foam to provide sufficient attenuation such that the amplitude of sounds produced in one chamber were at an amplitude in the adjacent chamber that is below the hearing threshold for *P. pustulosus*. A chorus of calls of *P. pustulosus* was played for 30 minutes using Altec-Lansing VS2320 speakers placed in each chamber and connected to a Dell 2350 computer that played the sound files. Vocalizations made by the focal male were recorded on a Juster MP-018 microphone attached to a Dell 2320 computer running the Linux operating system. The SoX (sox.sourceforge.net) open source sound utility software program was used to save the input from the microphones to digital sound files; vocalizations produced by the focal male were identified manually using the CoolEdit (www.cooledit.com) sound editing program. Males who did not call the first night were tested for as many as five consecutive nights before being disqualified; most males called during the first or second attempt. Thirty males from each rearing group were successfully recorded. Thirty calls were averaged for each male from the beginning, middle, and end of his calling activity. The temporal and spectral characteristics of these calls were automatically analyzed using programs written in the Signal (Engineering Design, Berkeley, CA) sound analysis environment. The characteristics of all 30 calls for each male were averaged to produce a single value for each of five call characteristics (duration, rise time, fall time, initial frequency, final frequency). Overall call duration, measured in milliseconds, is the total time of the call. The rise time describes the time from the beginning of the call to the point at which the call reaches maximum amplitude, and the fall time is the time from maximum amplitude to the end of the call. The initial frequency is the spectral frequency at the beginning of the call, and the final frequency describes the frequency at the end of the call. The data were evaluated first with a multivariate analysis of variance to test for effect of replicate. The data were then analyzed using multivariate analysis of variance using a Tukey's *post hoc* test for pairwise comparisons. All statistical tests were conducted using SPSS 15.0 (SPSS Inc., Chicago, IL).

Female preferences.—Based on the results from first experiment on male calls, a second experiment was designed to test the threshold for female preferences. Female *P. pustulosus* from the isolation-reared group and the group reared hearing calls of *P. enesefae* were tested for their discrimination of calls with longer or shorter duration times. Females from these two groups were chosen because the males of those groups produced the calls most different in duration. Calls of *Physalaemus pustulosus* were synthesized using the mean call characteristics of the males reared hearing the chorus of *P. enesefae*. These calls were approximately 270 ms in duration, 50 ms longer in duration than calls produced by naïve-reared males. To test the threshold for discrimination, calls that were 245 ms (25 ms longer) and 230 ms (10 ms longer) were also synthesized. These calls were paired with synthetic calls 220 ms in duration, the same duration

Table 1. Acoustic Properties of Calls Made by Male *Physalaemus pustulosus* Reared in Four Early Experience Treatments and from Studies of Wild Frogs. Mean, standard error (SE), and 95% confidence interval (CI) of temporal and three spectral properties of the calls of males reared hearing a chorus of conspecific *P. pustulosus*, in acoustic isolation, hearing a chorus of heterospecific *P. enesevae*, or hearing noise. Each of the 30 males in the early experience treatment groups is represented by a sample of 30 calls from that male. Data for wild-caught males was obtained in Panama (Ryan and Rand, 2003).

Early experience group		Initial frequency (Hz)	Final frequency (Hz)	Duration (ms)	Rise time (ms)	Fall time (ms)
<i>P. pustulosus</i>	Mean	943.071	483.268	265.948	35.655	230.293
	SE	3.31	6.82	10.77	6.21	11.08
	CI	6.48	13.46	21.11	12.16	21.73
Isolation	Mean	940.209	497.936	222.418	26.795	195.621
	SE	3.76	7.87	11.27	5.12	10.63
	CI	7.37	15.42	22.09	10.04	20.83
<i>P. enesevae</i>	Mean	936.578	488.525	274.994	36.485	238.509
	SE	3.28	4.86	8.21	6.70	8.96
	CI	6.43	9.53	16.09	13.31	17.57
Noise	Mean	938.131	498.594	258.781	30.908	240.871
	SE	2.37	5.68	10.17	6.66	9.93
	CI	4.65	11.12	19.93	13.05	19.47
Wild	Mean	1000.660	466.520	325.880	42.490	283.380
	SE	5.94	2.15	1.99	1.71	2.18
	CI	11.64	4.22	3.90	3.35	4.28

as the calls produced by the isolation-reared males. The calls of the males from the *P. enesevae* reared group were not significantly different in duration from the calls of the males in the *P. pustulosus* and noise rearing groups.

Twenty-eight adult females reared in isolation and reared hearing calls of *P. enesevae* (described above) were injected with 250 IU of human chorionic growth hormone to simulate the hormonal state of wild females found in amplexus (Lynch et al., 2006). These females were tested in a 2.7 × 1.8 meter acoustic chamber equipped with an infrared camera for observation. Calls were presented antiphonally from two speakers placed at the long ends of the chamber. Each call was repeated every two seconds, which is the typical call rate for two calling male *P. pustulosus*, and played at a sound level of 81 dB SPL (20 μmPa). Each female was placed in the center of the chamber and held under a funnel for two minutes while the stimuli were presented, then allowed to move freely. Choices were scored when the female moved from the center within five minutes, continued to move at least every two minutes, and came within 10 cm of one speaker within 15 minutes of being released. Each female was initially tested for their motivation to mate using conspecific simple and complex calls. If a female did not make a choice in this test of conspecific calls, she was not tested further. Female choices for each group were compared to the null hypothesis with an exact binomial probability. Choices among groups were compared with a binomial exact test. Choices were also tested for replicate position using a logistic regression.

RESULTS

Male calls.—For the temporal and spectral characteristics of male calls, there was no main effect of replicate position ($F = 1.206$, $df = 15$, $P = 0.266$), but there was a main effect of treatment ($F = 1.830$, $df = 15$, $P = 0.030$; summarized in Table 1). The initial frequency of calls was slightly different

among rearing groups but this difference was not significant ($F = 1.961$, $df = 3$, $P = 0.124$). The final frequency of calls produced by males in the four rearing groups was not significantly different ($F = 1.356$, $df = 3$, $P = 0.260$).

The duration of the calls among rearing groups was significantly different ($F = 5.817$, $df = 3$, $P = 0.001$; Fig. 2). Males reared in isolation produced calls that were shorter in overall duration than the calls produced by males who were reared hearing the chorus of calls of *P. pustulosus* ($P = 0.016$), the chorus of calls of *P. enesevae* ($P = 0.002$), and noise ($P = 0.005$). Overall call duration consists of the rise time, the time it takes for the call to reach maximum amplitude, and fall time, the time from maximum amplitude to the end of the call. The rise times were not significantly different across the rearing groups ($F = 0.523$, $df = 3$, $P = 0.667$). The fall times of the calls were different among rearing groups ($F = 4.238$, $df = 3$, $P = 0.007$); the fall times of the calls of males reared in isolation were shorter than those of males reared hearing the chorus of *P. enesevae* ($P = 0.018$) and the noise ($P = 0.011$) and close to significantly different than the calls of males reared hearing the conspecific chorus ($P = 0.081$).

Female preferences.—More females chose the call that was 50 ms longer in duration ($P = 0.007$; Fig. 3). Females showed no preference for calls that were either 25 ms ($P = 0.999$) or 10 ms longer than calls of *P. enesevae* ($P = 0.839$). There were no significant differences in female choices between treatment groups or interaction between replicate position and treatment for the 50 ms longer calls ($X_2 = 4.444$, $df = 4$, $P = 0.349$), the 25 ms longer calls ($X_2 = 8.653$, $df = 4$, $P = 0.700$), or the 10 ms longer calls ($X_2 = 3.965$, $df = 4$, $P = 0.411$; $X_2 = 3.706$, $df = 4$, $P = 0.100$ to $P = 0.411$).

DISCUSSION

Males that experience some acoustic stimulation develop typical mating calls regardless of the nature of that stimulation. Lack of early experience does, however, alter details of the advertisement call of male *P. pustulosus* in a

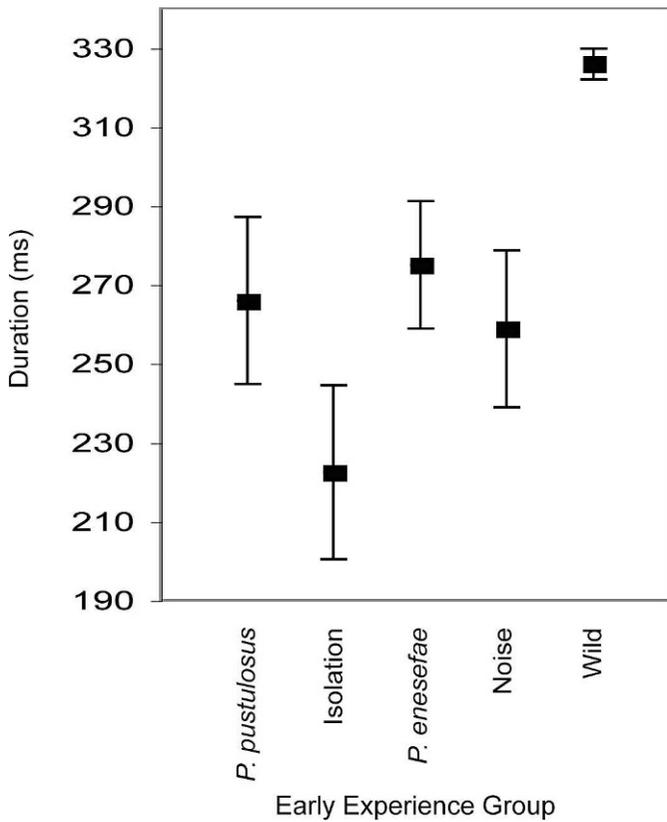


Fig 2. The mean overall call duration of advertisement calls produced by males reared in four acoustic treatment groups—*P. pustulosus* chorus, isolation, *P. enesefae* chorus, and noise—and of calls produced by wild males recorded in Panama. (Bars represent 95% confidence intervals.)

manner that is salient to female *P. pustulosus*. Male Túngara Frogs isolated from any calls or noise produced calls with a shorter overall duration. Call duration is one of the factors used by females to choose a mate and shorter calls are less attractive to females, demonstrating that experience may contribute to relevant variation in the advertisement calls used in female mate choice in this species. Juvenile Túngara Frogs have been observed at the ponds in which conspecific and heterospecific adults are engaging in mating behaviors (M. J. Ryan, pers. comm.), but no thorough studies have determined the precise movements of juveniles from metamorphosis to adulthood.

These results support the assumption that frogs do not require experience with conspecific tutors to acquire their calls. The large-scale deficits in acoustic signals observed in isolation-reared songbirds were not observed in any of the treatment groups in this experiment, and the calls of the males reared with heterospecific tutors were no different than those of the males reared hearing their own species calls. If experience does not play a major role in the acquisition of mating behaviors in the male of this species, an alternative explanation is that genetics play a strong role in the acquisition of calls in anurans. Although anecdotal experience with lab-reared frogs suggests that naïve-reared animals can acquire species-typical behavior, field research shows no correlation between signal variation and genetic distance (Heyer and Reid, 2003; Pröhl et al., 2006), suggesting that genes involved in the production of calls may not be under strong selection at the population level.

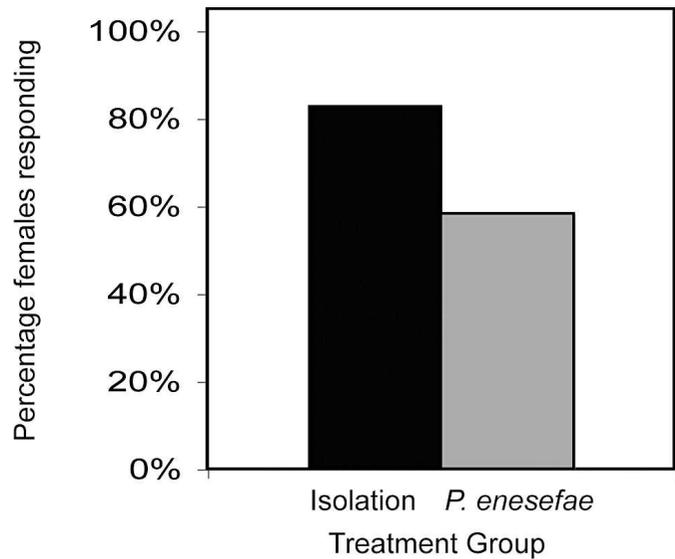


Fig 3. Percentage of *P. pustulosus* females responding to an advertisement call 50 ms longer in duration. Females were reared either in acoustic isolation or hearing a chorus of *P. enesefae*.

Further research is needed to clearly identify the genes and neural pathways involved in production of male mating behavior to confirm the role of genetics.

The temporal and spectral features of the calls of the males studied differ from those of males studied in Panama (Ryan and Rand, 2003) but not from those reported in a study on the variation of male calls across geographical distribution (Pröhl et al., 2006). The frogs used in our study were bred from adults originally from the region of Gamboa, Panama, and the calls characteristics for all frogs were within the range reported for Túngara Frogs from this locale by Pröhl et al. (2006), with the exception of the call duration and fall time for males reared in isolation. The differences seen with respect to Ryan and Rand (2003) may be due to variance in factors such as body size, temperature, humidity, naïvity, and reproductive status, which may affect the calls of this species. Evoking calls in the laboratory using conspecific playbacks may induce changes in calls, as demonstrated with *Hyla cinerea* (Penna et al., 1992). However, recordings of wild male Túngara Frogs are often made near active choruses, and these recordings are used to describe species-typical calls. The question of how early experience affects male calling that we address in this study is one that requires the use of lab-reared animals.

There was a treatment effect on the duration of the calls. The change may not be the result of any specific experience, but of the consequences of insufficient acoustic stimulation. The typical calls made by the heterospecific-tutored males and the noise-reared males suggest that a specific structure of the acoustic stimuli is not necessary to elicit normal call development in Túngara Frogs. Instead, there may be a threshold of minimum acoustic stimulation necessary for the auditory peripheral and central neural processes to develop. Although the isolation-reared frogs were not raised in absolute silence, they had an overall lower level of acoustic stimulation than the frogs in the other three rearing groups. In starlings reared with heterospecific tutors, males still developed stereotypical conspecific song (Boehner and Todt, 1996). Similarly, acoustic stimulation in Túngara Frogs may trigger an innate mechanism by which

normal calling behavior then develops. In this case, the groups reared hearing calls of *P. pustulosus*, calls of *P. enesefae*, and noise met some threshold of stimulation, whereas the acoustically isolated males, although not kept in absolute silence, had overall less acoustic input. Increased acoustic stimulation produces calls of longer duration in a leptodactylid frog (Penna et al., 2005). In contrast, song deprivation affects the expression of an enzyme critical to proper development of the neural circuitry in zebra finches (Sakaguchi and Yamaguchi, 1997). Acoustic stimulation interacts with gonadal hormones to produce normal vocal centers in canaries (Bottjer et al., 2004). To better understand if similar mechanisms are operating in frogs, future studies should evaluate the hormone and neurological changes in acoustically deprived anurans.

The effects of acoustic deprivation on male calls, whatever the mechanism, appear to be sufficient to influence female mating call preferences. Females reared in the same four acoustic treatment groups used in this study consistently preferred the longer calls produced by males that were not acoustically isolated. This discrimination against the shorter calls produced by acoustically isolated males could lead to reduced reproductive success in those males.

These data provide empirical support that anurans do not require experience with conspecific behaviors to acquire their species-typical advertisement calls. Genetic determination may play a larger role in the acquisition of male calls.

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