Evoked Vocal Responses Change with Experience in Male *Physalaemus pustulosus*

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The acquisition of signals used in intrasexual communication is not well studied. To better understand the possible contribution of early experience to the acquisition of intrasexual responses, we reared Neotropical Túngara frogs, *Physalaemus pustulosus,* in four acoustic environments: 1) conspecific chorus, 2) heterospecific chorus, 3) acoustic isolation, and 4) noise. For the heterospecific chorus, we chose the calls of a congener, *P. enesefae,* with which *P. pustulosus* is sympatric in parts of its distribution. We measured the evoked vocal responses (EVR) of these frogs in response to calls of the conspecifics, to calls of the congener, and to calls representing an intermediate between the two species. Male *P. pustulosus* reared hearing the calls of *P. enesefae* produced more calls overall and more complex calls in response to the call of *P. enesefae*. This is consistent with the hypothesis that the EVR of male anurans can be influenced by early experience and is the product of an interaction between genes and the environment.

SIGNALS used in mate recognition can also function in intrasexual competition (Andersson, 1994). In male–male competition, signals from one individual often evoke signals from neighboring males. In organisms that rely on acoustic signals, such as frogs and toads, the response of a male to the calls of neighboring males is known as an evoked vocal response (EVR). Male anurans may base their detection of and response to signals on proximity of neighbors or acoustic space. Male *Acris crepitans* are more likely to respond to close neighbors (Wagner, 1989), perhaps by using call amplitude (Wilczynski and Brenowitz, 1988). Just as females use a complex mix of cues when they express mate preferences, male frogs react to a complex mix of call characteristics when choosing which neighbors to which to respond (Greenfield and Rand, 2000). Males may be more selective in their response when heterospecifics are present to avoid responding inappropriately (Gerhardt, 1994).

Evoked vocal responses of individual males vary in nature and strength. For example, in a study of EVR in Emilio's Ground Frog, *Eupsophus emiliopugini,* males increased the frequency of calling as the amplitude of the signal increased but a few individuals also increased the complexity of their calls (Penna et al., 2005). Studies of EVR in male Túngara frogs, *Physalaemus pustulosus,* show variation around a mean (Ryan and Rand, 1998). Both results show that EVRs vary across individuals. If the source of this variation is genetic, then such variation should be heritable. If the variation is caused by experience, then individuals raised in different environments should have different responses. Genetics and environmental experience can interact to produce a complex source of variation. Understanding sources of variation can shed light on both the history and future directions of sexual selection, can provide information on differences between mating behaviors in congener or allopatric species, and help explain the benefits and mechanisms of mating systems (Jennions and Petrie, 1997).

In the Neotropical frog, *P. pustulosus,* males produce either simple advertisement calls (whine) or complex calls that consist of the simple call with added suffixes (chucks). The simple call consists of an amplitude modulated, downward frequency-sweep (Ryan, 1980). The escalation from simple to complex calls is facultative and females prefer calls with added chucks (Ryan, 1980; Rand and Ryan, 1981). Frog-eating bats preferentially prey on males making complex calls (Ryan et al., 1982; Tuttle et al., 1982).

Male *P. pustulosus* frogs increase calling when presented with conspecific simple calls and increase the production of chucks presented with conspecific calls (Ryan, 1985). Males do not typically increase calling when presented with calls of the congener *P. enesefae* but do increase calling in response to the calls of other species, including other congeners (Bernal et al., 2007).

The use of artificial stimuli can be useful in determining the nature of responses in *P. pustulosus*. Females respond in a continuous, not categorical, manner to signals that vary from the conspecific call. This was shown by presenting females with signals that vary along acoustic transects between the conspecific call and calls of heterospecifics (Ryan et al., 2003). Female response was stronger with the similarity between artificial stimuli and the conspecific call, with no apparent categories of perception. The call intermediate between *P. pustulosus* and *P. enesefae,* representing the point at which the acoustic properties differ enough that females no longer respond, can be used to test discrimination in male and female *P. pustulosus*.

To date, few studies have examined the mechanisms that contribute to the acquisition of male EVR (Gerhardt and Huber, 2002; Wells, 2007). The hormone arginine vasotocin is known to produce variation in calling behaviors of adult male *P. pustulosus* (Kime et al., 2007), but the role, if any, of this hormone during early development is not known. As with other behaviors, these EVRs may be acquired in means dictated primarily by genetics, by early experiential environment, or through an interaction of genes and environment. Early experience has been shown to alter male advertisement calls in *P. pustulosus* (Dawson and Ryan, 2009). Males reared in isolation (hearing no anuran sounds) produced calls that were shorter in duration, and such calls were less attractive to females, suggesting that some acoustic experience may be required for males of this species to produce attractive calls.

In this study, we test the hypothesis that gene by environment interactions also alter the EVR of male *P.*

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Table 1. Male-evoked Vocal Responses to Test Series Presented to Male *P. pustulosus* Reared in Four Different Acoustic Environments: 1) Reared Hearing *P. pustulosus,* 2) Reared Hearing the Congener *P. enesefae,* 3) Reared in Acoustic Isolation, and 4) Reared Hearing Noise.

Test series	*P. pustulosus*-reared males		*P. enesefae*-reared males		Isolation-reared males		Noise-reared males	
	Change in calls	Change in chucks	Change in calls	Change in chucks	Change in calls	Change in chucks	Change in calls	Change in chucks
P. pustulosus simple call vs. *P. pustulosus* complex call	1.18	2.40	3.05	4.00	3.03	1.12	0.73	3.08
Noise vs. *P. pustulosus* simple call	3.85	9.85	2.23	12.68	−0.10	6.53	0.73	6.95
Noise vs. intermediate call	1.53	6.40	5.35	17.28	−0.98	7.83	−2.70	3.93
Noise vs. *P. enesefae* call	−8.25	−2.63	0.38	5.65	−5.55	1.18	−10.25	−2.78

pustulosus. We raised male *P. pustulosus* in four acoustic treatments to evaluate the possible contribution of early experience in the acquisition of male response to acoustic signals.

MATERIALS AND METHODS

We reared *P. pustulosus* in sound-attenuated enclosures approximately 32 cm wide, 60 cm long, and 18 cm tall. The acoustic treatments in each enclosure were either: 1) *P. pustulosus* chorus, 2) *P. enesefae* chorus, 3) sound isolation, or 4) noise low-pass filtered at 5 kHz. Speakers broadcast the playback for conditions 1 and 2 at 80 dB SPL, the amplitude of a calling male measured at 1 meter, for 12 hours during each night. The noise was broadcast at 80 dB SPL for 24 hours. All animals were maintained in these treatments from tadpole stage until sexual maturity (approximately 24 mm SVL, 8–10 months of age). Following metamorphosis, all standing water was removed; *P. pustulosus* will not call except from standing water, so the lack of such prevented males in the enclosures from calling.

At sexual maturity, we studied male-evoked vocal responses by placing each male in a small sound-attenuated enclosure (22 cm wide, 24 cm long, 40 cm tall) equipped with a small pond of water from which to call. Each male acclimated in the test enclosure for approximately 30 minutes. Stimuli were played at 81 dB SPL from an Altec-Lansing VS2320 speaker driven by a Dell 2350 computer. Vocalizations produced by the male were recorded on a directional Juster MP-018 microphone to a Dell 2320 computer. The sox sound utility was used to capture the sound files which were then manually evaluated using the CoolEdit (Syntrillium) software program.

We presented 20 males with each of four test series. Each test series consisted of a pair of stimuli presented in this order: 60 seconds of control stimulus, 60 seconds of silence, 60 seconds of experimental stimulus, 60 seconds of silence, and 60 seconds of control stimulus. Each test series lasted five minutes, followed by five minutes with no playback. We varied the order in which the test series were presented to males. In each test series, the control stimulus was repeated to gain a more accurate measurement in case of variation in male calling activity. Males that did not call in each stimulus presentation of each test series were eliminated; most males called during the entire experiment.

The test series consisted of pairs of control and experimental stimuli (Table 1; Fig. 1). The four pairs were a) the *P. pustulosus* simple call paired with the *P. pustulosus* complex call, b) noise modulated with the amplitude envelope of the

P. pustulosus simple call paired with the *P. pustulosus* simple call, c) noise paired with an intermediate call that represents the threshold at which female *P. pustulosus* recognize the conspecific call versus the call of the congener *P. enesefae,* and d) noise paired with the call of *P. enesefae.*

For each male, we had five-minute recordings corresponding to each of the four test series. For each test series, we counted the total number of calls and the total number of chuck suffixes produced during each stimulus presentation. We calculated the per-minute rate of calls and the rate of chucks during presentation of the control stimulus and subtracted this from the per-minute rate of calls and chucks during presentation of the experimental stimulus. The resulting value is positive if the male increased activity during the presentation of the experimental stimulus. Three evaluators analyzed each sound file, and all evaluators were blind to the males' acoustic treatment. These methods are the same as those used in previous studies of EVR in anurans (see Ryan and Rand, 1998).

The data are summarized in Table 1. Each experiment is represented by the rate of change in calls (the number of calls produced per minute during the experimental stimulus minus the number of calls per minute during the control stimulus) and the rate of change in chucks.

The data were analyzed using analysis of variance to test for main effects of treatment (SPSS 15.0) using analysis of variance with a Tukey's *post hoc* test for pairwise comparisons, with Bonferroni adjustment for multiple comparisons.

RESULTS

Overall, there was an effect of early acoustic experience ($F = 1.639$. $df = 24$, $P = 0.036$). In the test series that paired the noise with the intermediate call, early acoustic experience had a significant effect on the rate of call production ($F = 3.161$, $df = 3$, $P = 0.029$; Fig. 2) and the rate of chuck production ($F = 6.570$, $df = 3$, $P = 0.001$; Fig. 3). In the test series that paired noise with the *P. enesefae* call, early acoustic experience had a significant effect on the rate of call production ($F = 5.915$, $df = 3$, $P = 0.001$; Fig. 4) and the rate of chuck production ($F = 4.268$, $df = 3$, $P = 0.008$; Fig. 5).

In the test series that paired noise with the intermediate call, males reared hearing *P. enesefae* calls produced more calls in response to the intermediate call than did males reared hearing noise ($P = 0.030$). Males reared hearing *P. enesefae* produced more chucks than did males reared hearing *P. pustulosus* ($P = 0.007$), reared in isolation ($P = 0.027$), or reared hearing noise ($P = 0.001$). Males in the
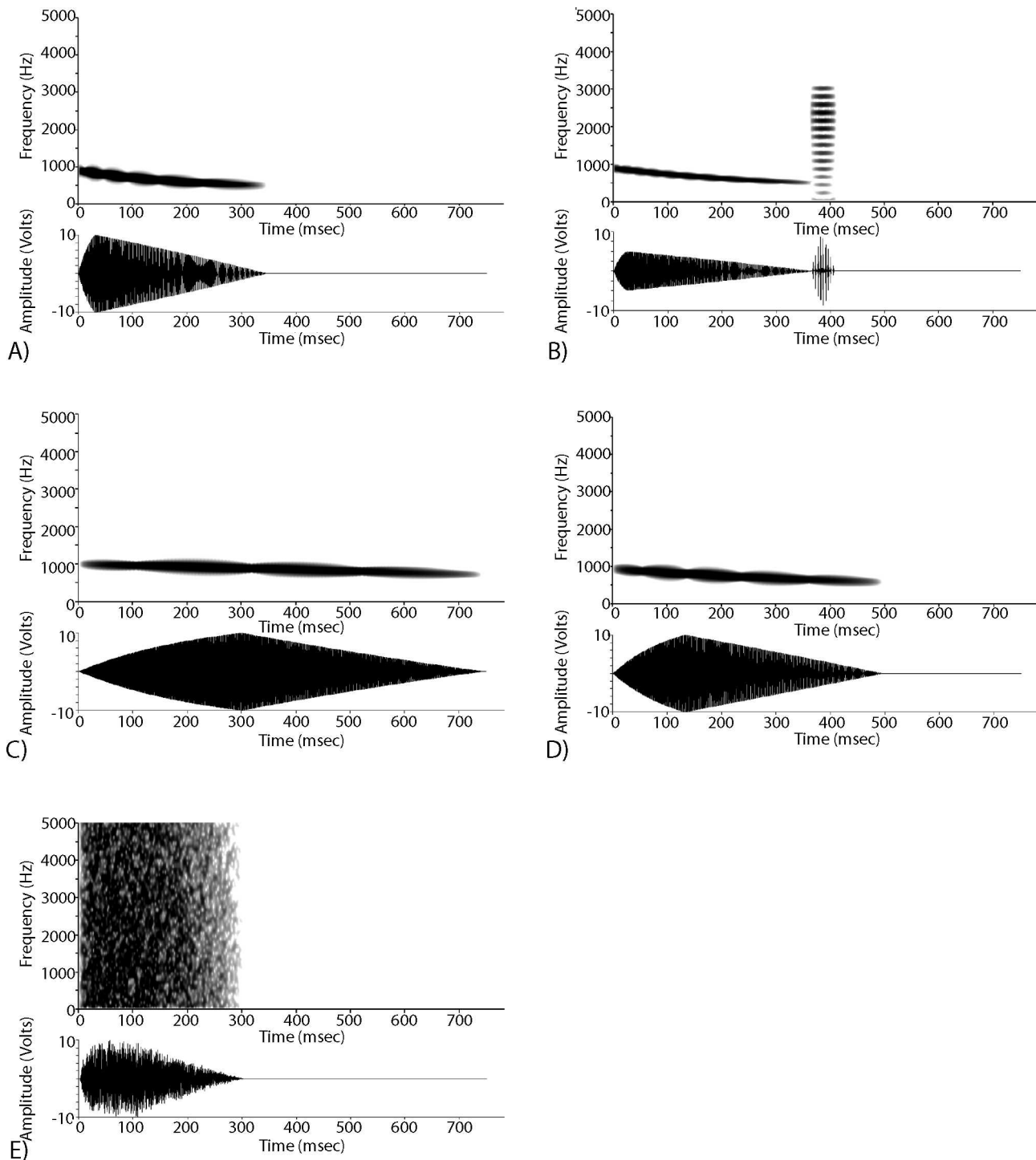
Fig. 1. Stimuli used for the tests of male-evoked vocal response: (A) simple *P. pustulosus* call; (B) complex *P. pustulosus* call with one chuck; (C) call of the congener *P. enesefae;* (D) intermediate call with acoustic characteristics between *P. pustulosus* and *P. enesefae* (see text); and (E) amplitude-modulated noise.

P. enesefae rearing condition consistently increased the rate of production of calls and of chucks when presented with the intermediate call.

In the test that paired noise with the call of *P. enesefae,* males reared hearing *P. enesefae* calls produced more calls in response to the *P. enesefae* experimental stimulus than did males reared hearing *P. pustulosus* ($P = 0.012$) or reared hearing noise ($P = 0.001$). Males reared hearing *P. enesefae* produced more chucks than did males reared hearing *P. pustulosus* ($P = 0.019$) or hearing noise ($P = 0.016$). In this test, males reared hearing *P. enesefae* increased the production of calls when presented with *P. enesefae* calls, while males in all other treatment conditions decreased calling activity.

Early acoustic environment did not alter production of calls and chucks in the other test series. In the test series that paired the conspecific simple call with the conspecific complex call, early acoustic experience had no significant effect on the rate of calls ($F = 0.851$, df $= 3$, $P = 0.470$) or the rate of chucks ($F = 0.804$, df $= 3$, $P = 0.496$). In the test
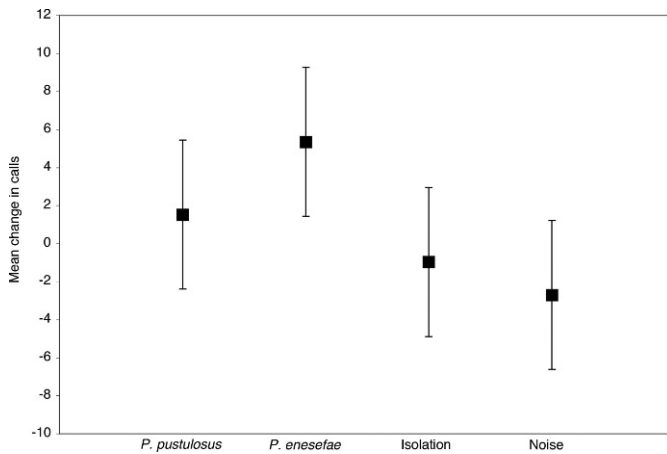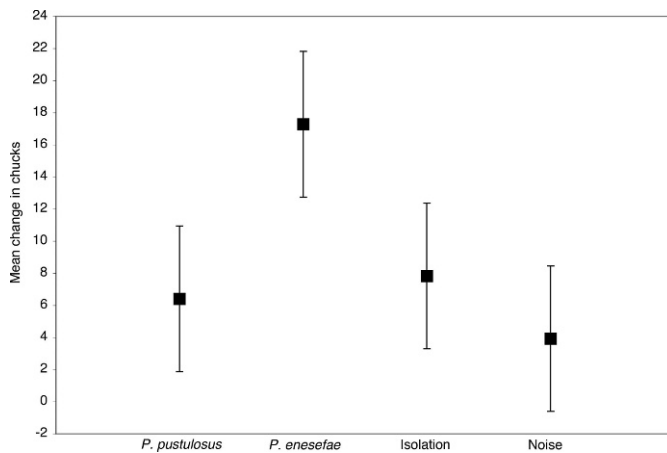
Fig. 2. Change in call production when the control stimulus "noise" is paired with the experimental stimulus "intermediate" call for all four early acoustic experience groups: *P. pustulosus*-reared, *P. enesefae*-reared, isolation-reared, and noise-reared. A positive number indicates an increase in calling to the intermediate call. *P. enesefae*-reared males increased calling more than males reared in other conditions, and this difference is significant when the *P. enesefae*-reared group is compared to the group reared hearing noise. Bars represent 95% confidence intervals.

series that paired noise with the conspecific simple call, early acoustic experience had no significant effect on the rate of calls ($F = 0.771$, df = 3, $P = 0.514$) or the rate of chucks ($F = 1.597$, df = 3, $P = 0.197$).

DISCUSSION

These results show that early experience can alter the EVR in male *P. pustulosus*. In particular, males reared hearing the calls of a congener, *P. enesefae,* were more likely to produce calls and chucks when presented with the *P. enesefae* call or a call intermediate between *P. enesefae* and *P. pustulosus*. Male *P. pustulosus* exhibit considerable variation in their vocal responses. We show here that early experience, such as the presence of congeners, may be responsible for some of this variation.
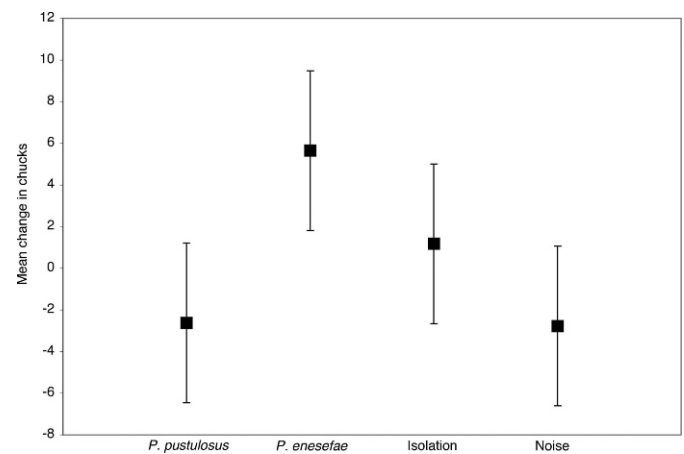

Fig. 3. Change in production of chucks when the control stimulus "noise" is paired with the experimental stimulus "intermediate" call for all four early acoustic experience groups: *P. pustulosus*-reared, *P. enesefae*-reared, isolation-reared, and noise-reared. *P. enesefae*-reared males produced more chucks than frogs reared in other conditions, and this difference is significant when the *P. enesefae*-reared group is compared to all other groups. Bars represent 95% confidence intervals.
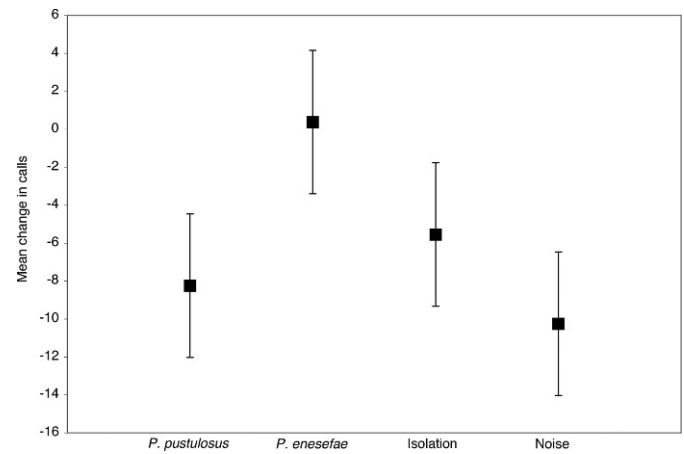
Fig. 4. Change in production of calls when the control stimulus "noise" is paired with the *P. enesefae* experimental stimulus, for all four early acoustic experience groups: *P. pustulosus*-reared, *P. enesefae*-reared, isolation-reared, and noise-reared. *P. enesefae*-reared males produced more calls than frogs reared in other conditions, and this difference is significant when the *P. enesefae*-reared group is compared to the *P. pustulosus*-reared and noise-reared groups. Bars represent 95% confidence intervals.

The frogs reared hearing *P. enesefae* calls produced a strong evoked-vocal response to the intermediate calls. This result is consistent with the hypothesis that the EVR of male *P. pustulosus* is acquired by an interaction of genes and environment. In other words, the *P. pustulosus* frogs tested in this experiment had the experience of being reared in an acoustic environment in which they only heard *P. enesefae*. The interaction of the genetics and experience may have led them to react strongly to an intermediate call, a call that shares acoustic characteristics of both species.

The increase in the production of chucks by *P. pustulosus* reared hearing *P. enesefae* may represent not only a source of variation but also a source of increased predation risk for certain individuals. Frog-eating bats attend particularly to the suffix chucks, so individual frogs producing more chucks

Fig. 5. Change in production of chucks when the control stimulus "noise" is paired with the *P. enesefae* experimental stimulus, for all four early acoustic experience groups: *P. pustulosus*-reared, *P. enesefae*-reared, isolation-reared, and noise-reared. *P. enesefae*-reared males produced more chucks than did frogs reared in other conditions, and this difference is significant when the *P. enesefae*-reared group is compared to the *P. pustulosus*-reared and noise-reared groups. Bars represent 95% confidence intervals.

are likely to suffer greater predation. If male *P. pustulosus* exhibit this behavior in areas of allopatry with *P. enesefae,* those individuals may be at greater risk of predation.

In *P. pustulosus,* early experience appears to influence male behaviors but not female preferences. In a previous study, the preferences of female *P. pustulosus* were not influenced by early experience (Dawson and Ryan, unpubl.). Male advertisement calls varied slightly with experience; males that were acoustically isolated produced shorter calls that were less preferred by females (Dawson and Ryan, 2009). The larger role of experience in producing variation in male behaviors may be due to stronger selection against female permissiveness and part of a larger trend of sex differences in receiver permissiveness (Bernal et al., 2007).

In this study, male *P. pustulosus* reared hearing conspecifics decreased the production of calls and chucks when presented with the *P. enesefae* stimulus, but they did continue to produce some calls and chucks. This is in contrast to data on the vocal responses of *P. pustulosus* in which males did not respond at all to the calls of *P. enesefae* (Bernal et al., 2007). That study used wild-caught males from Panama, a population that is not sympatric with *P. enesefae*. In Venezuela, *P. pustulosus* is sympatric with *P. enesefae*. Males in this area that exhibit variation in their vocal response might be exposed to altered mating opportunities or altered predation risk. Future studies should look at possible population-level differences in the vocal responses of male *P. pustulosus,* comparing individuals from areas of allopatry and sympatry for the influence of early experience on their vocal responses.

The tendency of experience to influence male vocal responses could also have implications for range expansion. New regions of sympatry with other species, combined with a heightened vocal response to those new species, could broaden the region where the energetic and predation costs are present.

In summary, vocal responses of male frogs can change with early experience. Male *P. pustulosus* reared hearing the calls of heterospecific males increased the rate of calling to those calls. Experience may influence acoustic communication in anurans and play a larger role in the variation of these behaviors than previously understood.

ACKNOWLEDGMENTS

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