



Local preference encoded by complex signaling: mechanisms of mate preference in the red-eyed treefrog (*Agalychnis callidryas*)

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Abstract

The Central American red-eyed treefrog (*Agalychnis callidryas*) is a nocturnal/crepuscular frog that exhibits high population-level phenotypic variation in a suite of traits generally important in anuran communication. Females of this species demonstrate a preference for local over non-local males, although the contribution of different communication modes to mate choice remains untested. We performed eight sets of experiments at La Selva Biological Station (Costa Rica) to test the relative roles of male advertisement call and flank-stripe pattern on female mate choice. For each trial, we used pre-recorded calls for acoustic signals and/or 3D-printed, hand-painted models for visual stimulus. Females selected conspecifics over heterospecifics when presented with either acoustic or visual stimuli only, indicating that both evolve as species-specific signals used for mate discrimination. Bayesian modeling showed that females chose local males over non-locals based on unimodal; responses to multimodal signals varied. Female mate choice in this species is likely nuanced and subject to the constraints of the local environment in which animals are signaling. Our data show that at least two traits that vary among populations are important for mate selection.

Significance statement

Evolution shapes animal communication in diverse ways to accommodate complicated signaling contexts, including environmental noise, the presence of eavesdroppers, and changing landscapes. Among species that use complex signals or signals in multiple modalities, understanding the role of signaling traits in reproductive behavior and their concomitant shifts between populations can shed light on lineage divergence, speciation, and the forces shaping communication. We used choice tests with *Agalychnis callidryas* to determine which traits lead to local-male mate preference. We found that both call and stripe are each sufficient to allow this nocturnal frog to choose mates in absence of the other stimulus. Multimodal signals were marginally better than call—but not color—alone. This may be a result of statistical noise or preliminary evidence of asymmetric use of traits across a complex landscape. These results suggest that call and color may encode both redundant information and multiple messages to receivers in this species: either is sufficient to elicit mate choice (redundant), but together, they enhance local mate preference (multiple messages). Further tests on how other communication modalities inform behavior will add to our understanding of complex signaling and lineage divergence among differentiated populations in this species.

Keywords Communication · Multimodal signaling · Phenotypic variation · Population divergence

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Introduction

The perception of communication signals across modalities has been a focus in behavior since the time of Darwin (Darwin 1871, 1872; Higham and Hebets 2013). Recent work demonstrates that behaviors associated with communication are likely far more complicated and nuanced than previously imagined. Indeed, many species from diverse lineages use complex traits, whether within a single modality and/or in multiple modalities, simultaneously or sequentially (reviewed in Candolin 2003; Partan and Marler 2005; Hebets and Vink 2007; Higham and Hebets 2013; Starnberger et al. 2014a, b). Such complexity may be explained in part by signal theory: the use of multiple cues can increase the likelihood of a signal reaching a receiver, and/or increase the complexity of the information encoded in a signal (Candolin 2003; Hebets and Vink 2007). Despite increasing appreciation of the prevalence and importance of complex signals, historically, many studies have sought to make predictions about perception, behavior, fitness, and evolution based on only a single receiver (e.g., males in agonistic encounters) in a single population with an assumption that results apply broadly across populations. However, there is growing appreciation that interpopulation variation in signaling strategies and preferences exist (Madden 2006; Ward and McLennan 2009; Bailey and Zuk 2012; Auld et al. 2016; Barbosa et al. 2016; Hardy et al. 2017; Symes 2018). Further, the strength, direction, and patterns of selection that underpin trait evolution can differ across isolated and divergent populations, as well as across social contexts (e.g., mate recognition, alarm calls, competition). Without understanding the complexities that mediate trait evolution, we are limited in our ability to infer the consequences of signal variation for population divergence in behavior (Uy and Safran 2013).

Understanding the mechanisms that mediate complex behaviors such as mate choice can provide insight into the causes and consequences of accrued lineage divergence and potentially into reproductive isolation. The use of complex signals in mate choice, which often hinges on the display of multiple traits, frequently in multiple modes (Partan and Marler 1999; Candolin 2003), could impact the strength of sexual selection for each trait and/or suite of traits. For example, if complex signals reduce mate-choice errors, selection on at least one signal should be relatively strong, accelerating the accumulated divergence among populations (West-Eberhard 1983). In contrast, selection would be relatively weak if the relative value of a trait differed across the landscape, or if there is high variance in mate preference (reviewed in Candolin 2003). Because a signal's utility is constrained by a receiver's ability to perceive it (Weaver 1953), information contained in signals may lose or shift function, with potential consequences for species and mate recognition if those preferences do not shift synchronously (Lande 1981; West-Eberhard 1983; Pomiankowski and Iwasa 1998; Panhuis et al. 2001;

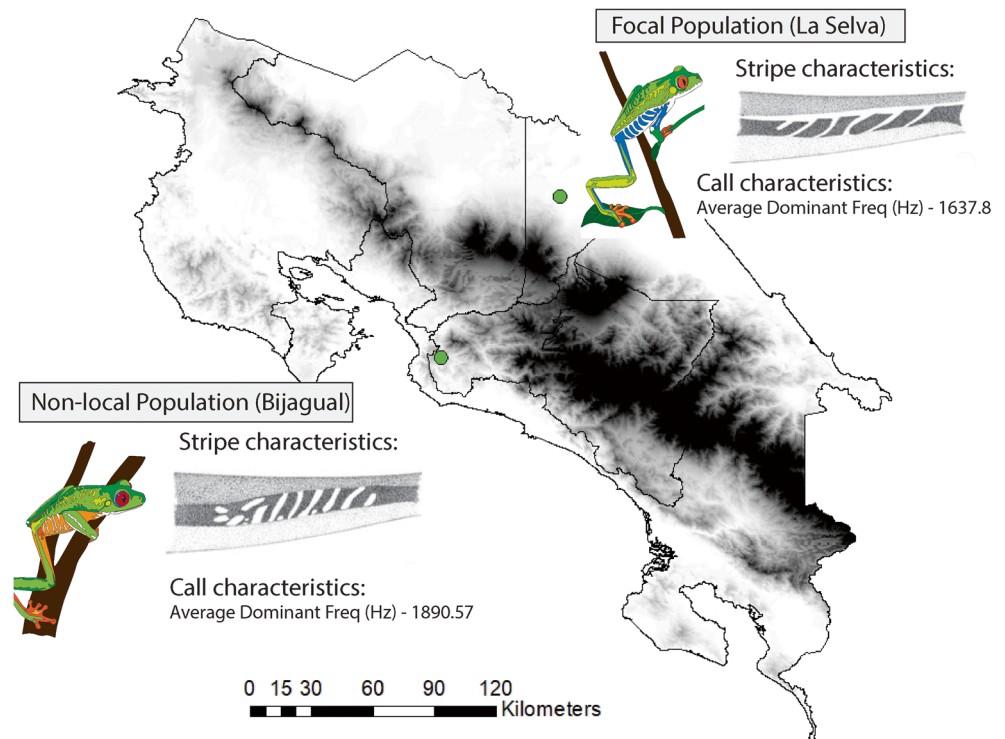
Candolin 2003). Further, social context can shape the emphasis that a receiver places on a cue (Candolin 2003). Thus, populations could diverge across a suite of traits important in communication through the combined effects of selection and drift, with the strength of selection on those traits (whether single or combined) mediating the evolution of reproductive isolation among differentiated populations/lineages.

Anuran amphibians have long been a focus of communication and mating behavior studies because they are easily studied in the laboratory and the field, they respond well to manipulative behavioral trials, and many species are minimally disturbed by human observers (Starnberger et al. 2014b). Anurans are also a viable group for studies on complex signals due to the mounting evidence that cues other than acoustics are important in communication, including chemical, seismic, and visual cues (Duellman and Trueb 1986; Narins et al. 2003; Taylor et al. 2008; Caldwell et al. 2010; Wells 2010; Starnberger et al. 2013, 2014b). For example, green treefrogs (*Hyla cinera*) are more responsive to multimodal playbacks with both visual and acoustic components than to unimodal ones (Laird et al. 2016). In addition, the anuran vocal sac, which produces sound, also serves as a tool for visual, and even chemical signaling (Preininger et al. 2013a; Starnberger et al. 2014a).

The red-eyed treefrog (*Agalychnis callidryas*) is a broadly distributed, crepuscular/nocturnal Neotropical frog, ranging from southern México to Colombia (Savage 2002). Within Costa Rica, red-eyed treefrogs show pronounced regional phenotypic differentiation in a number of traits generally important in anuran mate choice, including male advertisement call, female courtship behavior, body size, flank and leg color pattern (Fig. 1), and skin peptides (Duellman 1970; Robertson and Robertson 2008; Robertson and Zamudio 2009; Robertson et al. 2009; Robertson and Vega 2011; Davis et al. 2016; Akopyan et al. 2017). In nature, females approach calling males that may be obscured by dense vegetation, suggesting that acoustic cues serve to locate potential mates. However, observations of courtship behaviors with live animals suggest that females select males based on more than simply acoustic traits (Jacobs et al. 2017), raising the possibility that visual cues, such as the presence and characteristics of the flank stripe, are also important for this species (Robertson and Greene 2017). Finally, for some populations of Costa Rican and Panamanian red-eyed treefrogs, females have a weak but significant preference for local males (Akopyan et al. 2017; Jacobs et al. 2017; Robertson et al. 2018), indicating that differences in social signals are discernable and meaningful. However, the mechanisms underlying mate assessment and choice by female *A. callidryas* remain unstudied.

We conducted a series of experiments to disentangle the relative roles and interactive effects of complex signals in mate choice for red-eyed treefrogs. We first demonstrated that call and stripe pattern are important social signals in mate choice by testing female choice between conspecific calls/color patterns

Fig. 1 Focal populations of *Agalychnis callidryas* within Costa Rica, Central America. All experiments were conducted at La Selva Biological Station (Heredia Province, 10.425646°N, 84.022056°W) using females from that locale. Non-local frogs were modeled after frogs from a population at Bijagual (Puntarenas Province, 9.730249°N, 84.569364°W). The two allopatric populations are separated by the Cordillera de Talamanca (in black). Average dominant frequency of advertisement calls from each population (Akopyan et al. 2017) and stripe characteristics are shown. Stripe pattern images modified from Robertson and Robertson (2008)



and heterospecific cues. Next, we hypothesized that both male advertisement call and stripe pattern contribute to female preference for local males, and that while each would be sufficient to elicit female mate choice when presented alone, mate choice for local males would be stronger if together these signals encode more information or make a signal more likely to be detected. We chose these traits not in expectation that they are the only cues that females assess when selecting mates, but rather as a first step in understanding how receivers assess and choose mates in the context of unimodal and complex signals in this species. We used stimuli from two allopatric Costa Rican populations that demonstrate high levels of phenotypic and genotypic differentiation (Robertson et al. 2009; Fig. 1). These populations also show low levels of both premating (Jacobs et al. 2017) and postzygotic reproductive isolation (JMR and KK unpublished data).

Methods

We studied red-eyed treefrogs at La Selva Biological Station, a lowland tropical rainforest in Heredia, Costa Rica (Fig. 1) from June to August 2016, coinciding with the red-eyed treefrog breeding season (Savage 2002). To test the relative role of call and stripe pattern on mate choice in red-eyed treefrogs, we conducted female mate-choice experiments using a combination of acoustic playbacks and visual models (see below). Female mate-choice behavior has been described in previous studies and is easily observed and scored in this species (Pyburn 1970;

Akopyan et al. 2017; Jacobs et al. 2017). We collected gravid females from breeding aggregations (the presence of eggs is readily detectable in gravid females of this species). All females were tested the night they were collected and released at the same ponds the next day. Females that released eggs prior to testing were excluded from trials. Because of the video setup, we were unable to record our data blind to treatment.

Stimulus preparation

To create sound files for acoustic playbacks, we used pre-recorded male advertisement calls from wild populations. The heterospecific calls in experiment 1 were obtained from recordings of *Tlalacohyla loquax* in Belize (Kaiser et al. 2011). We chose *T. loquax* calls because they share two key characteristics with calls of red-eyed treefrogs: short duration and broadband frequency content. The two species are syntopic throughout Central America (Duellman 1970; Savage 2002). We created files for conspecific calls from recordings of males from local (La Selva) and non-local (Bijagual, Puntarenas Province) populations for experiments 2–8 (Fig. 1). Bijagual red-eyed treefrogs produce calls with a significantly higher mean dominant frequency than frogs from La Selva (Akopyan et al. 2017). The description of how the acoustic stimuli were created has been detailed elsewhere (Akopyan et al. 2017); briefly, we used Audacity (v. 2.0.6) to create stimuli comprising single-note calls from both species, with a call rate mimicking the natural mean call rate for red-eyed treefrogs at this site (2.8 min^{-1}). We isolated one call per recording, using a total of 28 local calls and 18 non-

local calls. All tracks were the same length and total power (RMS) was normalized in Adobe Audition (CS5.5). Tracks were reused in an experiment only after all others were used.

For visual models, we created a model of a red-eyed treefrog in perching posture based on digital photos, and then constructed plasticine models *via* a 3D printer. We hand-painted four model types (Table 1) using paint mix that matched the hue, saturation, and brightness measurements obtained from digital photographs that were color corrected from a standard gray–white–black card in Adobe Photoshop (CC2015). We considered a match to have values that are within 2.5° (hue) and 10% (for saturation and brightness). We selected a generalized stripe and color pattern that represented each focal population: blue flanks with no stripes for the heterospecific model, closely resembling the syntopic *A. saltator* (experiment 2); blue flanks with local stripes (experiments 2, 4–8); blue flanks with non-local stripes (experiments 4, 6–7); and orange flanks with non-local stripes (experiment 8) (Robertson and Robertson 2008). Although flank-stripe pattern is unique to each individual frog in natural populations, there are general species and population differences in terms of stripe thickness, shape, and number (Robertson and Robertson 2008): local (La Selva) frogs have fewer, wider vertical flank stripes that are often connected by a horizontal line at the intersection of the green dorsum and blue flank, whereas the non-local Bijagual frogs have many narrow vertical flank stripes with no horizontal stripe (Fig. 1) (Savage and Heyer 1967; Robertson and Robertson 2008). By using the same blue background for experiments 2, 4–7, we removed the possible confounding effect of hue on mate selection, and instead focused on the role that population-specific stripe pattern plays in mate choice. We used a single synthetic exemplar for each experiment painted to match the mean of each population. We opted for a single model in order to minimize the chance that subtle and unintentional variation in how the stripes were painted would swamp out any signal of population-specific trait preference. Models were placed on disks attached to small motors (VEX Robotics, Greenville, TX) creating movement to attract females' attention (Paluh et al. 2014; Robertson et al. 2018). Motors were wrapped in cotton and foam baffling to minimize acoustic and vibrational cues and placed inside a plastic container with a small hole in the lid for a post that connected motors to platform disks. Disks turned with the following looping program: repeat twice at a speed of 100° s^{-1} : (spin -20° , wait 20 s, spin 20° , wait 20 s); spin 45° at 50° s^{-1} ; repeat twice at 50° s^{-1} : (spin -40° , wait 25 s, spin 40° , wait 25 s); spin 90° at 50° s^{-1} , wait 30 s, spin -90° at 50° s^{-1} , wait 60 s. This movement was not intended to mimic the natural movements of frogs.

Experimental design

We conducted all trials in a 1-m^2 experimental enclosure created from mesh cloth stretched over a metal rod skeleton

(Robertson et al. 2018). We used fine metal mesh (1 mm) on all sides but one, which was covered in a coarse plastic mesh (10 mm). All experiments were conducted using a two-choice design. A cloth partition created a modified Y shape at the back half of the enclosure. A tape line was laid 10 cm from the back corners where stimuli were presented, which was used to record when females approached the models and/or speakers. The chamber was housed in an outdoor ambient-temperature laboratory with a roof. The laboratory was in the forest, protecting it from anthropogenic noise and light. No additional light was introduced and trials were recorded with a video camera (Bell and Howell DNV16HDZ Night Vision Camcorder, Wheeling, IL) on the infrared setting.























Models were placed at the opposite end of the enclosure from focal females, in the modified-Y portion of the enclosure, approximately 15 cm above the ground. For experiments with acoustic stimuli, speakers (Pignose 7-1000, La Vegas, NV) were placed immediately behind the enclosure and acoustic stimuli were played back from iPod Minis (Apple Corporation, Cupertino, CA). Speakers were calibrated with an SPL meter (Radio Shack SPL Meter 33-00099, Fort Worth, TX) to play calls back at approximately 68 dB SPL at 1 m, similar to measured red-eyed treefrog calls at La Selva (KK and JMR unpubl. data). For trials with both acoustic and visual signals, the models were placed inside the enclosure, ~ 2 cm in front of the speakers. We alternated the enclosure side for presentation of stimuli to avoid side bias.

Females were acclimated in the chamber in a covered plastic tub at the midpoint of the front end of the chamber nearest the camera. The acclimation period lasted 2 min, during which a speaker (Pignose 7-1000, La Vegas, NV) suspended above the chamber broadcast pre-recorded frog chorus sounds from an iPod Nano (Apple Corporation, Cupertino, CA). Acclimation broadcasts included local conspecific calls and were calibrated to play at approximately 65 dB SPL (Radio Shack SPL Meter 33-00099, Fort Worth, TX). After the acclimation period, the chorus playback was changed to a recording that did not include conspecific calls (i.e., was recorded at a pond where *A. callidryas* do not breed; this recording included *Dendropsophus ebraccatus*, *D. microcephalus*, *T. loquax*, and *T. picta*) to prevent inadvertent directional stimuli while still providing natural aggregation sounds (Dapper et al. 2011). Acoustic playback of experimental stimuli began (where relevant) and the female was released from the acclimation chamber in equidistant from the stimuli.

Each trial ran for 10 min. We documented female choice of a stimulus when the female performed one of the following courtship behaviors: a flank display (female displaying a side toward the model), a back display (positioning back oriented toward model within 50 cm of model), and/or an approach within 10 cm of the model (Akopyan et al. 2017). Females that failed to make a choice were excluded from analysis. To ensure that

Table 1 Experimental design and sample sizes. We conducted eight experiments using different model frogs and recorded calls. Local = *Agalychnis* from the study site, La Selva, Heredia Province. Heterospecific = *Talcohyla loquax*. No Stripe = painted as local without

flank striping. Non-local = Bijagual, Puntarenas Province. *n* represents the total number of females included in analyses. Females not making a choice in trials were excluded from analysis

Experiments 1 & 2. Is call or stripe pattern a species-level recognition trait?			
Stimulus	Male 1	Male 2	n
Experiment 1: Call			
			16
	Heterospecific call (<i>T. loquax</i>)	Local conspecific call	
Experiment 2: Stripe pattern			
			20
	No stripe	Local stripe	
Experiments 3–5. Is call or stripe pattern sufficient to elicit choice of local over non-local mate?			
Stimulus	Male 1	Male 2	n
Experiment 3: Call only			
			19
	Non-local Call	Local Call	
Experiment 4: Stripe pattern only			
			19
	Non-local Stripe	Local Stripe	
Experiment 5: Call vs. Color			
			23
	Local Stripe (No Call)	Local Call (No Model)	
Experiments 6–8. Does signaling in multiple modes encode more information to elicit choice of local mate?			
	Male 1	Male 2	n
Experiment 6: Mismatched cues			
	 	 	57
	Non-local Stripe, Local Call	Local Stripe, Non-local Call	
Experiment 7: Multiple cues			
	 	 	50
	Non-local Stripe, Non-local Call	Local Stripe, Local Call	
Experiment 8: Multiple Cues including color			
	 	 	29
	Non-local Color and Stripe, Non-local Call	Local Stripe, Local Call	

females were not tested multiple times, we photographed the left and right flanks of all females (Canon Powershot SX710HS, Melville, NY). Red-eyed treefrogs have unique stripe patterns, which allowed us to create a reference catalog of tested females against which we checked each new individual.

Female choice trials

We examined the mechanisms of female mate choice through eight experiments (Table 1), each with $n = 16$ –53 trials, for a total of 229 trials in which females made choices. Across all trials, females failed to make a choice in approximately 20% of trials. Sample sizes for each experiment, including total number of females tested per experiment, are listed in Table S1.

Experiments 1–2. Is advertisement call or stripe presence a species-level recognition cue in mate choice? Females were presented with cues in only a single communication mode: acoustic or visual. These experiments tested whether females discriminate and choose conspecific over heterospecific stimuli. In experiment 1 ($n = 16$), frogs were presented with a choice between local conspecific and heterospecific calls without visual models present. In experiment 2 ($n = 20$), females were presented with models without the broadcast of conspecific calls. One model was painted with local conspecific stripes; the other was an identical model lacking the characteristic stripes of conspecifics (but closely resembling a syntopic congener).

Experiments 3–5. Is local call or stripe pattern sufficient to elicit local mate choice? We tested how call and stripe pattern impact local preference. Females were presented with local or non-local conspecific calls (experiment 3; $n = 19$) or models with local or non-local conspecific stripe patterns (experiment 4; $n = 19$). In experiment 5 ($n = 23$), we tested which signal elicited stronger local-mate choice: females were presented with a local call with no visual stimulus on one side of the enclosure and a local visual model on the other with no acoustic stimulus.

Experiments 6–8. How do call and color pattern interact to elicit local mate choice? Complex signals can encode more information—whether it is redundant or increased information—than unimodal signals. In experiment 6 ($n = 57$), we tested the relative strength of each cue in mate-choice behavior by mismatching cues: females were given a choice between a model with local stripes paired with a non-local call, or non-local stripes and local call. In experiment 7 ($n = 50$), we tested whether female preference for local males was stronger when multiple cues in different modes were presented than when a single cue was presented, as in experiments 3–5. Females were presented with models with local stripes paired with local calls and non-local stripes paired with non-local calls. In experiment 8 ($n = 29$), we included flank hue to test whether adding a color signal improved local mate choice. The experimental setup was identical to experiment 7

except that the non-local model had orange flanks to mimic its actual coloration in the wild.

Data analyses

For all experiments, we used Baye's theorem to calculate the posterior probability distribution for the choice of the local male by each female (or with respect to stripe pattern in the case of both choices being local). The posterior probability distribution, specified as the beta distribution, was calculated analytically by virtue of using the beta distribution (the conjugate prior distribution to a binomial likelihood) for prior information. Previous studies of red eyed tree frogs of female choice of local or non-local males in this same population by our laboratory (Akopyan et al. 2017; Jacobs et al. 2017) provided estimates of prior probabilities of local male choice for experiments 3–5, 7, and 8. For experiments 1, 2, and 6, we used an uninformed prior distribution in which the shape parameters a and b of the beta distribution were set equal to 1. There are no suitable data for use as prior probability of local male choice for experiment 6 because antagonistically mismatched cues do not naturally occur, thus, prior data with either or both cues present confound estimates for mismatched cues. The previous studies by Akopyan et al. (2017) and Jacobs et al. (2017) only tested conspecifics, so no prior data were available for the experiments testing con- versus heterospecific cues (i.e., 1 and 2). We nevertheless compared outcomes for experiments 1 and 2 with and without informed priors (i.e., using the probability of choosing local over non-local and assuming these data represented a conservative estimate for the heterospecific case as an extreme non-local pattern) and the differences in the estimated modes of the posterior distributions averaged 0.036 (i.e., 3.6%) across both experiments. We calculated odds ratios based on estimated modes for comparing across experiments. Data were analyzed using R (version 3.1.2). Data are provided in ESM S2.

Data availability statement All data generated or analyzed during this study are included in this published article (and its supplementary information files; ESM S2).

Results

Experiments 1–2. Is advertisement call or stripe presence a species-level recognition cue in mate choice? Both call and stripe were effective cues in species recognition, with stripe pattern being slightly stronger (Fig. S1; Table 2). Females were approximately 1.33 times more likely to choose conspecifics over heterospecifics based on stripe alone than on call alone (Table 3).

Table 2 Results of Bayesian posterior probability modeling. For each experiment, we list the calculated mode, 95% CI, and probability of females choosing a local stimulus. See text for detailed justifications of prior distributions and calculations

Experiment	Mode (\pm 95% CI)	$p(\text{local})^a$
Experiments 1 and 2. Is call or stripe pattern a species level recognition trait?		
1. Call	0.750 (0.5200-0.910)	3.00
2. Stripe pattern	0.800 (0.601–0.931)	4.00
Experiments 3–5. Is call or stripe pattern sufficient to elicit choice of local over non-local mate?		
3. Call only	0.545 (0.347–0.734)	1.20
4. Stripe only	0.660 (0.529–0.777)	1.94
5. Call vs. stripe pattern	0.514 (0.359–0.667)	1.06
Experiments 6–8. Does signaling in multiple modes encode more information to elicit choice of local mate?		
6. Mismatched cues	0.456 (0.332–0.584)	0.84
7. Multiple cues	0.620 (0.519–0.714)	1.63
8. Multiple cues including color	0.657 (0.540–0.762)	1.92

^a $p(\text{local})$ = odds of choosing local stimulus except in experiments 5 and 6. In experiment 5, mode was arbitrarily calculated as choice for stripe. In experiment 6, it was calculated as choice for local stripe/non-local call

Experiments 3–5. Is local call or stripe pattern sufficient to elicit mate choice? Which signal do females attend to more when making mate choices? Both traits were informative in mate selection (Fig. S1; Table 2). Females provided with only models with local and non-local stripes were slightly more likely to choose a local male over non-local than were females provided with only auditory playbacks of local and non-local advertisement calls (Tables 2 and 3). When local call and stripe were presented as separate stimuli alone (experiment 5), females chose each stimulus with nearly equal probability (Tables 2 and 3; “local” choice defined arbitrarily as choice for stripe).

Experiments 6–8. How do call and color pattern interact to elicit local mate choice? In experiment 6, we arbitrarily

Table 3 Odds ratios for comparisons of experimental outcomes. Experiments used to calculate ratios given in parentheses

Comparison (experiments)	Odds ratio
Is call or stripe pattern a species-level recognition trait?	
Stripe: Call (2:1)	1.33
Is call or stripe pattern sufficient to elicit choice of local over non-local mate?	
Stripe: Call (4:3)	1.62
Does signaling in multiple modes encode more information to elicit choice of local mate?	
Multiple vs. single cue (call) (7:3)	1.36
Multiple vs. single cue (stripe) (7:4)	0.84
Multiple (with color) vs. single cue (call) (8:3)	1.60
Multiple (with color) vs. single cue (stripe) (8:4)	0.99
Multiple (with color) vs. multiple cues (8:7)	1.17

defined a “local” choice as choosing local stripe paired with non-local call. When cues were mismatched, females chose males similarly to experiment 5, where a single cue was presented (Table 2). Multiple, properly matched cues (experiment 7) increased the probability of females choosing a local male; adding non-local flank color to the non-local model slightly increased the probability further (experiment 8), although the confidence intervals for these estimates overlap substantially (Table 2).

Which cue(s) best predict female mate choice?

Odds ratios for the different experiments suggest that females presented with multiple cues were 1.36 times as likely to choose a local male than those presented with just a local call stimulus but 0.84 times as likely than those presented with a local stripe model. Adding non-local flank color in addition to stripe (experiment 8) only made females marginally more successful at discerning local males from non-locals than in experiment 7 (Tables 2 and 3).

Discussion

Tests of the mechanisms of female mate choice and local-male preference in red-eyed treefrogs revealed several key findings. First, females can, and do, make choices in low-light conditions (moonlight) solely based on visual cues, suggesting that visual traits can evolve through sexual selection. Second, advertisement call and flank stripe are each sufficient to elicit mate choice in female red-eyed treefrogs. Finally, call, stripe, and color appear to each have additive effects in mate choice: color appears to have the smallest effect, but call and stripe can work to either cancel (in the case of mismatched signals, experiment 6) or reinforce the effect of the other when in concert.

Based on advances in our understanding of anuran communication and mating behavior, it is not surprising that females attend to multiple signals to recognize conspecifics and choose mates; here, we demonstrate that females attend to at least two traits when choosing mates. We also contribute to a growing body of literature that shows that visual cues are important for mate choice, even for nocturnal frogs (Hödl and Amézquita 2001).

Vision in the dark

Others have documented the apparent use of visual cues in conjunction with acoustic cues in the nocturnal frog *Hyla arborea* (Gomez et al. 2009; Richardson et al. 2010; Gomez et al. 2011). To our knowledge, this is the first example of a nocturnal/crepuscular frog choosing a male in the absence of auditory stimuli. The strong discrimination (77% of trials) between the two visual models (that were otherwise identical

in size and shape, and that had identical coloration except for the presence of white vertical stripes) provides evidence that females possess the visual system to discern between a heterospecific (without stripes) and a conspecific (with stripes) model. Thus, it appears that the contrast between the dark flank and bright white stripes is both perceivable and meaningful as a social signal, as previously suggested based on a suite of ecological and phylogenetic analyses of this group (Robertson and Greene 2017). Whether the frog perceives population differences in hue (e.g., orange, purple, dark blue, light blue) as a difference in luminosity remains to be tested. We included hue as a variable in a subset of our trials (experiment 8) and found that hue increased the likelihood of females choosing local males, but not seemingly as much as stripe or advertisement call. However, we view these results as preliminary. The sensory environment in which hue is perceived by the receiver is likely to vary among habitats that differ in canopy and vegetative structure, and the sensory environment should be considered and tested (Maan et al. 2006).

Mate choice: unimodal vs. multimodal

That both call and stripe pattern are each sufficient to elicit mate choice suggests that the information encoded in these traits is at least partly redundant (Candolin 2003). Red-eyed treefrogs, like many frogs, select mates in noisy, dark, dense breeding aggregations, and information theory suggests that redundant signals are effective in these settings as they increase the likelihood of the message reaching the receiver (Shannon and Weaver 1949). Indeed, a number of other frog species rely on complex signals, whether uni- or multimodal, to encode redundant signals (Rand et al. 1992; Vásquez and Pfennig 2007; Preininger et al. 2013b).

We demonstrated that females can use both the call and color pattern when choosing between a heterospecific and conspecific signal and between conspecific signals as well (Table 2). Our results comport with previous studies demonstrating that La Selva females prefer local males. La Selva females were 1.94 times as likely to choose a local male model over a non-local based on stripe pattern, 1.20 times based on call, and 1.63 times as likely when they had both signals. The decrease in probability of local choice with multiple signals relative to visual signal alone has two possible interpretations. The first is one of simple statistical noise: the probabilities on which these odds ratios are based are similar and the 95% confidence intervals overlap considerably. Thus, it is possible that unimodal signals perform similarly to multimodal signals. A second, biologically relevant explanation lies in how red-eyed treefrogs search for mates. If females use cues sequentially—i.e., first using acoustic cues to localize appropriate males in a complex environment, and then using visual cues to inform choice (Robertson and Greene 2017)—this result would be expected. Females in experiments with models were

always within visual range, and the potential information added in a call may not be important. This possible asymmetric use of traits would imply that the information encoded in advertisement call and stripe pattern also provides multiple, non-redundant messages (Candolin 2003; Hebets and Papaj 2005). Nonlinear or other non-redundant interactions of traits have been observed in other anurans, including túngara frogs (*Engystomops pustulosus*), cricket frogs (*Acris* sp.), and the foot-flagging frog *Micrixalus saxicola* (Burmeister et al. 1999; Preininger et al. 2013a; Stange et al. 2017).

We interpret these combined results to suggest that the role these traits play in mate choice is subtle, nuanced, and complex. The local-male preference previously documented in various populations of *A. callidryas* suggests that there may exist a “local standard of beauty” that is bolstered by information encoded in more than one signal for females to discern (Jacobs et al. 2017; Robertson and Greene 2017). Frogs are known for using a diverse array of communication modalities and the information encoded therein for mate selection (e.g., Ramer et al. 1983; Ryan et al. 1992; Gerhardt et al. 1996; Wollerman 1998; Farris et al. 2002; Gerhardt and Huber 2002; Smith and Roberts 2003; Narins et al. 2006; Zhu et al. 2016; Cayuela et al. 2017). Studies with other anuran species have shown that adding complexity to stimuli or to experimental designs can alter female mate-choice behavior, including causing females to choose inferior stimuli (Lea and Ryan 2015; Tanner et al. 2017). We thus predict that multimodal signals and female choice based on these in *A. callidryas* may be modulated by other factors, including context, environmental noise, and signal complexity. We also predict that other traits may play a role in mate choice in this species, thus increasing the complexity of female mate choice. These questions will be the focus of future work.

Phenotypic differentiation and the evolution of premating reproductive isolation

We tested the mechanisms of mate choice to examine whether observed patterns of phenotypic differentiation among populations contributes to premating reproductive isolation, and thus is likely to accelerate lineage divergence. Despite the strong signal of mate choice for the conspecific call and color pattern relative to heterospecific signals, premating reproductive isolation at the species level was not complete: 22% of females courted the male with heterospecific stimulus (20% visual, 25% call). Moreover, courtship is not equivalent to mating: females may ultimately reject unsuitable males or males may reject females. Thus, it is possible that our study underestimates premating isolation among these populations. Taken together, these data are consistent with other studies of interspecific mate-choice trials (Arnold 2015; reviewed in Robertson et al. 2018). With respect to reproductive isolation at the population level, our results were concordant with our a priori

expectations: previous mate-choice experiments with live animals (rather than models) demonstrated female preference for local males, but that preference was not absolute. In that study, La Selva females were 1.86 times as likely to choose a local male than a non-local Bijagual male (compared to 1.92 times as likely in this study) (Jacobs et al. 2017). The fact that females in our study, which were presented with models, had a similar incidence of choosing a local male than a study that used live males that varied in many measurable traits (body size, skin peptides, behavior, color pattern, vocalization) suggests that the two signals we isolated (advertisement call and stripe pattern) captured the majority of the variation in traits that females assess when choosing mates. A caveat about the difference in our estimates of incidence-rate ratios between these two studies is that the live males in the previous study did not always call during trials, potentially weakening the observed effect of multiple signals in that study, suggesting still other traits are likely to play an important role in mate choice. Finally, even if local males confer higher mating success, overlapping trait variation for males in each population could mean that some non-local males still outperform some local males (Laird et al. 2016). Low levels of premating isolation would thus result in gene flow among divergent populations if they were to come into contact, the evolutionary consequence of which would be to slow the pace of lineage divergence.

Conclusion

Our motivations were two-fold: (1) to investigate mechanisms of female mate choice to examine the strength and interaction of multiple signals in a species of frog that breeds in low ambient-light conditions and (2) to understand whether evolved differences in social signals could have consequences for local mate choice and premating reproductive isolation among diverging populations. Both advertisement call and stripe pattern were sufficient for species recognition and for local mate choice.

In nature, females may assess any number of other traits and are clearly doing so in a highly complex sensory environment; the sensory noise within breeding aggregations is often considered a strong selective force for multisensory communication (Stange et al. 2017). In addition, relative selection pressures on different signaling modalities may be asymmetrical (Gomes et al. 2017). We have shown that both signals serve a social function but acknowledge that both vocalization and color pattern are likely to serve multiple functions, including male-male territorial displays and anti-predatory behavior; the transmission of signals in other social contexts could impact the evolution of these traits. How these factors apply to the evolution of communication within and between divergent and potentially isolated populations of *A. callidryas* will more completely inform the direction and pace of evolution.

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Compliance with ethical standards

Ethical approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted. This research was approved by the IACUC at CSUN (1516-015a) and conducted by permission from the Ministerio de Ambiente y Energía (SINAC-SE-GCUS-PI-R-061-2015).

Conflict of interest The authors declare that they have no conflict of interest.

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