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Melodic males and flashy females: Geographic variation in male and female reproductive behavior in red-eyed treefrogs (*Agalychnis callidryas*)

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Abstract

Geographic variation in courtship behavior can affect reproductive success of divergent phenotypes via mate choice. Over time, this can lead to reproductive isolation and ultimately to speciation. The Neotropical red-eyed treefrog (*Agalychnis callidryas*) exhibits high levels of phenotypic variation among populations in Costa Rica and Panama, including differences in color pattern, body size, and skin peptides. To test the extent of behavioral premating isolation among differentiated populations, we quantified male advertisement calls from six sites and female responses to male stimuli (acoustic and visual signals) from four sites. Our results show that both male advertisement calls and female behavior vary among populations: Discriminant function analyses can predict the population of origin for 99.3% ± 0.7 of males based on male call (dominant frequency and bandwidth) and 76.1% ± 6.6 of females based on female response behavior (frequency and duration of visual displays). Further, female mate choice trials (*n* = 69) showed that population divergence in male signals is coupled with female preference for local male stimuli. Combined, these results suggest that evolved differences among populations in male call properties and female response signals could have consequences for reproductive isolation. Finally, population variation in male and female behavior was not well explained by geographic or genetic distance, indicating a role for localized selection and/or drift. The interplay between male courtship and female responses may facilitate the evolution of local variants in courtship style, thus accelerating premating isolation via assortative mating.

KEYWORDS
anurans, courtship, lineage divergence, sexual selection

1 | INTRODUCTION

Behavior, like any phenotype, can vary geographically due to the influences of selection, gene flow, and drift (Foster & Endler, 1999). Adaptive behaviors may evolve due to natural and/or sexual selection and result in population variation when selective pressures vary across habitats. Sexual selection in particular can mediate population variation and diversification in courtship behavior, which can have strong consequences for premating reproductive isolation via assortative mate choice (West-Eberhard, 1983). Geographic variation in courtship signals (e.g., male advertisement call) and female preference for those signals can promote lineage divergence (Gerhardt, Dyson, & Tanner, 1996; Reynolds & Fitzpatrick, 2007; Maan & Cummings, 2008; Gade, Hill, & Saporito, 2016). Indeed, the role of behavioral

diversification in speciation is well documented (Coyne & Orr, 2004; Wilkins, Seddon, & Safran, 2013). Divergent sexual selection on male signals via female preference for those signals (Rodríguez et al., 2013) has been demonstrated in birds (Seddon et al., 2013), reptiles (Hendry, Guiher, & Pyron, 2014), fishes (Conte & Schluter, 2013), and insects (Oh, Fergus, Grace, & Shaw, 2012).

Anurans are a classical system for studying sexual selection for several reasons. First, female mate choice is common among species with a prolonged breeding season (Wells, 1977; Stebbins & Cohen, 1995). Second, courtship signals are generally apparent and easily observed and measured, with many frogs relying heavily on vocal and/or visual cues. In addition, many anurans come to breeding aggregations to attract and choose mates, so individuals are often conspicuous. Finally, courtship cues—especially male advertisement calls—often vary across populations (Wilczynski & Ryan, 1999; Bernal, Guarnizo, & Lüddecke, 2005; Boul, Funk, Darst, Cannatella, & Ryan, 2007; Velásquez et al., 2013). Indeed, many studies have quantified differences in female preference across populations in response to geographic variation in male signal (Ryan, Perrill, & Wilczynski, 1992; Summers, Symula, Clough, & Cronin, 1999; Pröhl, Koshy, Mueller, Rand, & Ryan, 2006; Pröhl, Hagemann, Karsch, & Hobel, 2007; Velásquez et al., 2013); however, no study to our knowledge has quantified population-level differences in how females exert that preference, not just which stimulus they prefer.

The reproductive behavior of red-eyed treefrogs was first described from a population in Veracruz, Mexico. Pyburn (1970) described female response behavior as a back display during which females approach a male head-on and turn 180°, soliciting males to mount onto their backs, indicating readiness for amplexus. We identified a second mating signal—termed a flank display—while conducting mate choice trials with live females and males (Jacobs, Vega, Dudgeon, Kaiser, & Robertson, 2016). When presenting a flank display, a female approaches a male either head-on or from the side, orients so that her side is facing him, and presents outstretched arms and legs, revealing colorful flank stripes. Flank displays last up to 600 s, occur within 30 cm of the male, and are often followed by back displays, which can result in amplexus.

Multiple lines of evidence support that color pattern (which is conspicuous during a flank display) serves as a social signal that could evolve through selection in red-eyed treefrogs. Red-eyed treefrogs exhibit substantial geographic variation on a relatively small spatial scale within Costa Rica and Panama (Figure 1), yet genetic analyses indicate ongoing gene flow among phenotypically differentiated populations (Robertson, Duryea, & Zamudio, 2009), and phylogenetic analyses of Central American phyllomedusines demonstrate that color pattern variation among lineages is not explained by evolutionary history (Robertson & Greene, 2017). Further, a mate choice study of two allopatric red-eyed treefrog populations demonstrated female preference for local males, even in the absence of calls (i.e., when males did not call during experiments) (Jacobs et al., 2016), suggesting that visual signals are important in mate selection. To date, there is no knowledge of population variation in male advertisement call and/or female response behaviors to male stimuli.

Combined, these findings laid the groundwork to explore the hypotheses that female red-eyed treefrogs choose local males based on population differences in both male call and color pattern and that both male and female behaviors vary geographically. We based the hypotheses regarding female behavior on our observation that females have a complex behavioral response to males that includes at least two postural displays, as opposed to simple phonotaxis as observed for many frogs. Additionally, we tested whether local and non-local male stimuli elicited different behaviors from females. Finally, we tested whether genetic and/or geographic isolation can explain differences in visual and acoustic mating signals among populations (Table 1; Figure 1; Robertson et al., 2009). If this is not the case, selection on male advertisement calls could act to generate local preferences. Our findings suggest that the interaction between male advertisement calls and female response is an important but overlooked component of anuran mating systems and may have consequences for lineage divergence by sexual selection.

2 | METHODS

2.1 | Field sampling

We conducted field surveys in 2014 from June to September, which coincides with the breeding season of the red-eyed treefrog. We sampled six populations of red-eyed treefrog in Central America (Figure 1). Caribbean and Pacific populations are allopatric, isolated by the Cordillera de Talamanca, a mountain range extending the length of the lower Central American continental divide. On each versant of the mountains, populations vary in the degrees of genetic and geographic isolation (Robertson et al., 2009; Robertson & Zamudio, 2009). We analyzed the advertisement calls of 6–41 males from each of six populations, and the mating displays of 15–20 females from a subset of four populations. Female behavior trials were not conducted in Manzanillo, CR or Gamboa, PA.

2.2 | Male advertisement calls

We recorded calling males in six natural populations (*n* = 6–41, Figure 1). Advertisement calls were recorded at a distance of 0.5–2.0 m using a digital recorder (Marantz PMD661, Mahwah, NJ) and directional microphone (AudioTechnica AT815b, Stow, OH; Sennheiser ME66/K6, Hanover, Germany; or Rode NTG-2, NSW, Australia). All calls were recorded at 16 bit 44.1 kHz sample rate. Calls from the Gamboa population were provided by M. Caldwell.

Males of *A. callidryas* have multiple call types. The most common advertisement call is a chock note that can be ornamented with a variable number of secondary notes (Duellman, 2001). While the role of complex calls in mate choice is unknown for *A. callidryas*, complex calls are preferred in other anuran species (Ryan & Rand, 1990; Boul et al., 2007). Therefore, we used only calls that comprised a single chock note. We analyzed at least one call per individual. In Raven (Charif, Clark, & Fristrup, 2003), we measured dominant frequency with the Peak Frequency function and interquartile bandwidth (bandwidth; the
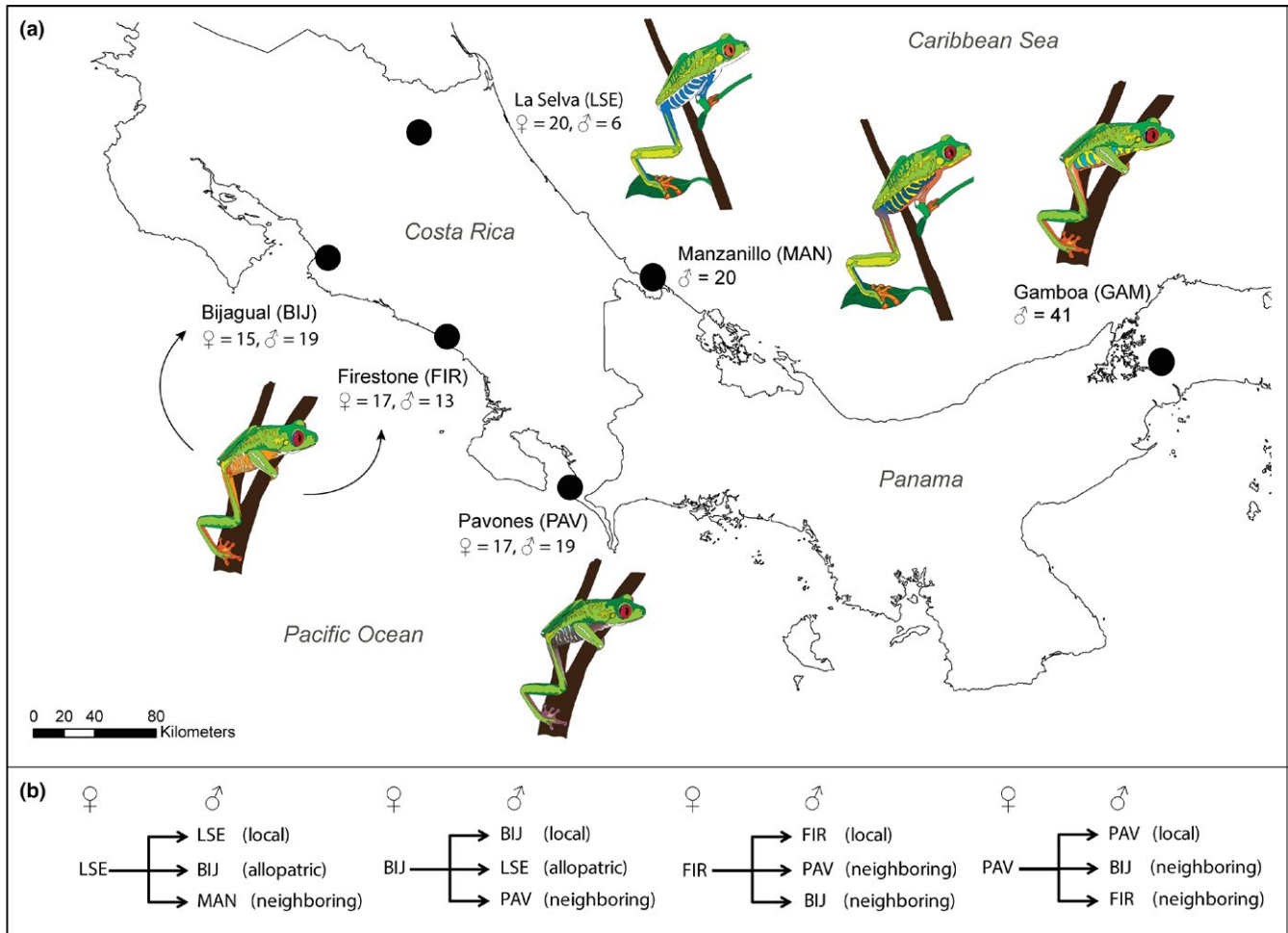
FIGURE 1 (a) Population localities from three biogeographic regions: eastern Costa Rica (La Selva Biological Research Station, Heredia province, CR, 10.473758°N, −84.021370°W; Manzanillo, Limón province, CR, 9.633517°N, −82.655632°W), western Costa Rica (Bijagual, San José province, CR, 9.739343°N, −84.558034°W; Firestone Reserve, Puntarenas province, CR, 9.274927°N, −83.858938°W; Pavones, Puntarenas province, CR, 8.389753°N, −83.136785°W), and central Panama (Gamboa, Panama, 9.123386°N, −79.693032°W). Sample sizes provided for male- and female-signal analyses. Note colorful flanks and differences among populations. (b) Experimental design for female courtship trials. Focal females from each of four populations were tested with a local male and two non-local males

frequency range containing the middle 50% of sound energy) with the IQR Bandwidth function.

We used a nested (individual within site) mixed-effects model to test for among-population differences, followed by unplanned pairwise comparisons. We used a k-nearest neighbor discriminant function analysis (DFA) to test whether call traits predict male population of origin. Recordings were also used as stimuli in behavior trials. We used multiple regression to model continuous response variables (dominant frequency and bandwidth) against three continuous predictor variables: geographic distance (km) and two measures of genetic distance (F_{ST} based on microsatellite and mtDNA markers; Table 1) (Robertson et al., 2009). Statistical analyses were conducted in R v. 3.2.5 (R Core Development Team 2016) using packages lme4 for fitting linear mixed-effects models and multcomp for Tukey's pairwise comparisons (Hothorn, Bretz, Westfall, & Heiberger, 2008; Bates, Maechler, Bolker, & Walker, 2015). Discriminant function analyses were conducted in StataIC v. 10.1 (StataCorp, 2008).

2.3 | Female behavior trials

Both amplectant and non-amplectant gravid females from four populations (n = 15–20, Figure 1) were collected for trials and were considered to be reproductively receptive because they were found in breeding aggregations. Females that released eggs prior to trials were not used in experiments. Each individual was photographed with a mobile phone camera (Apple iPhone 5s, Cupertino, CA). We used these images to identify individuals by the unique color pattern (vertical flank-stripe characteristics) to ensure we did not use a female in more than one set of trials. Frogs were released at the site of capture after trials each night.

Behavior trials were conducted in an experimental enclosure that was 1.2 × 1.2 × 1 m, constructed of 2-cm-diameter metal tubing, covered with thin metal screen (0.25 cm^2 opening), and a plywood floor. To minimize the effect of artificial light and noise and protect the experimental set-up from ambient weather conditions,

TABLE 1 Pairwise population differences in genotype (microsatellite and mtDNA) and phenotype (male advertisement call; dominant frequency and interquartile bandwidth) for six populations of red-eyed treefrogs. Measures of geographic and genetic distance obtained from Robertson et al., 2009

| Population contrast | Geographic dist. (km) | F_{ST} (msat) | F_{ST} (mtDNA) | Dominant frequency (Hz) | | Interquartile bandwidth (Hz) | |
				Z	p	Z	p
La Selva vs. Manzanillo	172	**0.10**	0.03	0.260	1.000	**−4.910**	**<.001**
La Selva vs. Bijagual	117	**0.21**	**0.91**	**−3.108**	**.021**	−0.428	.998
La Selva vs. Firestone	214	**0.23**	**0.95**	**−3.056**	**.025**	−0.019	1.000
La Selva vs. Pavones	316	**0.29**	**0.96**	2.461	.126	2.443	.133
La Selva vs. Gamboa	495	**0.20**	**0.85**	**−3.587**	**.004**	−2.610	.089
Manzanillo vs. Bijagual	272	**0.16**	**0.84**	**−5.429**	**<.001**	−0.435	.998
Manzanillo vs. Firestone	369	**0.20**	**0.91**	**−4.760**	**<.001**	−2.224	.215
Manzanillo vs. Pavones	471	**0.25**	**0.93**	**4.201**	**<.001**	**3.077**	**.024**
Manzanillo vs. Gamboa	330	**0.09**	**0.74**	**−7.154**	**<.001**	**−3.257**	**.013**
Bijagual vs. Firestone	113	**0.05**	**0.96**	0.275	1.000	2.535	.107
Bijagual vs. Pavones	213	**0.09**	**0.97**	−0.845	.956	**2.973**	**.033**
Bijagual vs. Gamboa	601	**0.18**	**0.90**	0.444	.998	**4.674**	**<.001**
Firestone vs. Pavones	101	0.04	**0.86**	−0.981	.918	0.123	1.000
Firestone vs. Gamboa	698	**0.25**	**0.95**	0.037	1.000	0.957	.927
Pavones vs. Gamboa	800	**0.30**	**0.96**	−1.400	.712	−0.943	.931

Significant differences for pairwise population comparisons are bolded (Tukey's contrasts, *p* < .05).

we constructed enclosures under awnings within 500 m of collection sites.

Females were presented with plasticine frog models (Figure 2) with recordings of male advertisement calls broadcast from behind the model. Each female was presented with a different male stimulus (McGregor, 2000). We used models in place of live male frogs to control for the role of male behavior in female choice, such as male advertisement call, chemical cues, movement, and courtship behaviors (Figure 2). We painted models to mimic the flank hue and stripe pattern of each population using paint that matched the hue, saturation and brightness (HSB) of color-corrected digital photographs of live frogs in Adobe Photoshop. Models were mounted on robotic disks (Vex Robotics, Greenville, TX) to provide a visual stimulus with movement. Movement of models was not meant to simulate the natural movement patterns of male *A. callidryas*. Rather, moving models provide an additional visual stimulus that results in a better estimate of natural response in frog behavior trials compared to stationary models (Paluh, Hantak, & Saporito, 2014). Robots were programmed using the Modkit software for Vex Robotics with the following looping program: repeat twice at a speed of 100° per seconds: (spin −20°, wait 20 s, spin 20°, wait 20 s); spin 45° at 50° per seconds; repeat twice at 50° per seconds: (spin −40°, wait 25 s, spin 40°, wait 25 s); spin 90° at 50° per seconds, wait 30 s, spin −90° at 50° per seconds, wait 60 s.

Acoustic stimuli were created using male calls recorded in natural populations (see above). A single call was isolated in Audacity (Mazzoni & Dannenberg, 2000) and used to generate a stimulus track with a call rate similar to the natural mean call rate (2.8 calls/min) of *A. callidryas* (Duellman & Pyles, 1983). Track length and power (total RMS) were equalized for all tracks in Audition (Adobe 2011). Stimuli were broadcast at levels similar to natural call levels in this species (65–70 dB SPL measured at 1 m; unpubl. data) from speakers (Pignose 7-1000, Las Vegas, NV) placed immediately behind plasticine models. Sound pressure levels were measured using a portable sound level meter (RadioShack 3389 2055, Fort Worth, TX).

We conducted trials between 1930 and 2330 h and used a no-choice design to quantify female response behavior (Wagner, 1998). Females were presented, in random order, with a model from the local population or one of two non-local populations (Figure 1b). At the start of each trial, we acclimated the female to the enclosure for 5 min under a clear plastic chamber because our preliminary observations indicated that females express increased escape behavior in the initial minutes of being placed in the enclosure. During the acclimation period, we broadcast a natural chorus of conspecific and heterospecific calls at 60–65 dB SPL (measured at 1 m) directly above the female acclimation chamber from a portable speaker (Pignose 7-1000, Las Vegas, NV). The acclimation playback was recorded at a fixed position in the Cantarana Swamp at La Selva Biological Research Station. The same acclimation recording was used for all trials to avoid inadvertent changes in cues that may be associated with different sites, choruses, or species compositions at different sites. The male model was obscured from view until the end of the acclimation period. At the start of each trial, the model was uncovered, the acoustic stimulus (a recording of a male *A. callidryas* call from one of six respective populations) behind the model was broadcast, and the female was released from the acclimation chamber. At this time, the acclimation playback was changed to a

FIGURE 2 Live male frog from La Selva on left; plasticine La Selva frog model on right. In *A. callidryas*, the shade of green on the dorsum can change with light exposure due to intracellular transport of pigment cells. Photograph of live male was taken during the day (light green dorsum). Frog model was painted to match the dorsum shade of an advertising male at night

recording of a breeding assemblage of frogs but lacking *A. callidryas* calls to avoid introducing a confounding directional cue. This playback was recorded at a site where several species that co-occur with red-eyed treefrogs bred, but red-eyed treefrogs did not. Thus, we could be sure that there were no inadvertent conspecific calls in the playback.

Trials ran for 10 min in ambient darkness and were visualized and recorded for later review with an infrared video camera (Bell and Howell DNV16HDZ Night Vision Camcorder, Wheeling, IL). Each female was used in three 10-min trials (one local and two non-local males, Figure 1b). Between trials, we housed females in opaque, soundproof containers. All trials for a given female were conducted on the same night. Females rested 5–40 min between trials. We scored the occurrence and frequency of female response behavior (back and flank displays) to each male stimulus. The duration of flank display was also recorded for each focal female; due to the uniformly short duration of back displays (<3 s), we did not measure duration for this trait.

We analyzed female displays using generalized linear mixed models for repeated measures with two fixed factors: female population and local vs. non-local male stimulus relative to female origin. Iteratively weighted least-squares means were used to estimate the probability of events followed by Tukey's pairwise comparisons. We used a k-nearest neighbor DFA to test whether population can be predicted based on mating displays. We used logistic regression to model categorical response variables against three continuous predictor variables: geographic distance (km) and two measures of genetic distance (F_{ST} based on microsatellite and mtDNA markers; Table 1) (Robertson et al., 2009). Statistical analyses of female behavior were conducted in R v. 3.2.5 (R Core Development Team 2016) using packages lme4 and car (Bates et al., 2015; Fox et al., 2016). Discriminant function analyses were conducted in StataIC v. 10.1 (StataCorp, 2008).

3 | RESULTS

3.1 | Male advertisement calls

Male calls varied among populations in mean dominant frequency and interquartile bandwidth (Hz) ($F_{5, 126}$ = 12.626, $p < .001$; $F_{5, 123}$ = 8.402, $p < .0001$, respectively; Table 1) and the DFA based on both bandwidth and dominant frequency accurately assigned all but one individual to natal populations (Table 2).

Examination of these two parameters (bandwidth and dominant frequency) revealed geographic patterns of calls: Differences in dominant frequency alone were associated with comparisons between allopatric populations (Table 1). Only two contrasts differed in interquartile bandwidth alone, both including BIJ (western Costa Rica). Central Panama (GAM), although phenotypically similar to eastern Costa Rica populations (Robertson & Robertson, 2008), had calls most similar to western Costa Rica (PAV, FIR). Finally, PAV (western Costa Rica) and LSE (eastern Costa Rica) calls did not differ significantly in either parameter despite the average male body size being largest at LSE and smallest at PAV of all sites (Robertson & Robertson, 2008). Despite this result, males from these sites were correctly assigned to natal populations based on the DFA that included both call parameters.

Geographic and genetic (microsatellites and mtDNA) distances did not predict divergence in male advertisement calls (Table 3). For dominant frequency, the intercept-only model was 4.0 times better than any model including a predictor variable. For interquartile bandwidth, the intercept-only model was 3.6 times better than any model including a predictor variable.

3.2 | Female behavior trials

The probabilities of both back and flank displays varied among populations, but females performed more flank displays than back displays overall (Figure 3). For back display, La Selva and Bijagual show population-level differences in female response behavior regardless of male stimulus, whereas for flank display, Pavones and Bijagual show population-level differences (Figure 3). Females were also more likely to perform a back display to local male stimuli (ranging from 1.4-fold more likely at Bijagual to 2.2-fold at La Selva; best-fit log-linear model). We further detected population-level differences in behavior using DFA: the occurrence of back/flank displays and time in flank display resulted in correct classification of most females to their source population (Table 2). The majority of misclassified females from LSE, BIJ, and FIR (between 20% and 28%) were all incorrectly assigned to PAV, whereas over 93% of females from PAV were correctly assigned to their natal population, with only two individuals (4%) incorrectly assigned to FIR.

Geographic and genetic (microsatellites and mtDNA) distances were weak predictors of the occurrence of back/flank displays (Table 4). For flank display, the intercept-only model was 2.1 times

| True site | Classification | | | | | | |
	La Selva	Bijagual	Firestone	Pavones	Gamboa	Manzanillo	n
La Selva	100	0	0	0	0	0	8
	61.67	3.33	6.67	28.33	–	–	60
Bijagual	0	100	0	0	0	0	37
	0	77.78	0	20	–	–	45
Firestone	0	0	100	0	0	0	15
	0	0	71.43	28.57	–	–	42
Pavones	0	0	0	95.65	0	4.35	23
	0	0	4.44	93.33	–	–	45
Gamboa	0	0	0	0	100	0	109
	–	–	–	–	–	–	–
Manzanillo	0	0	0	0	0	100	47
	–	–	–	–	–	–	–

TABLE 2 Male advertisement calls (top values) and female courtship behavior (bottom values) can be statistically classified into natal populations

Diagonal values (in gray) indicate percentage correctly classified. Males were classified based on mean dominant frequency and bandwidth (Hz). Females were classified based on combined variables (occurrence of back/flank display, time invested in flank display). Female behavior was not tested at Manzanillo or Gamboa for this study.

TABLE 3 Geographic and genetic distances between populations fail to predict variation in male advertisement calls. Values calculated for the model selection for multiple regression analysis using three continuous predictor variables: geographic distance (km) and two measures of genetic distance (F_{ST} based on microsatellite and mtDNA markers) and two continuous response variables of male call: dominant frequency (a) and interquartile bandwidth (b)

Model	Predictor variables	AICc	ΔAICc	AICc weight	Cumulative weight	Log-likelihood	Evidence ratio
(a)							
1	Intercept-only	199.96	0	0.55	0.55	−97.48	$\frac{Model\ 1}{Model\ 2}=3.96$
2	Geo	202.71	2.75	0.14	0.69	−97.26	
3	Msat	202.74	2.78	0.14	0.82	−97.28	$\frac{Model\ 2}{Model\ 3}=1.01$
4	mtDNA	203.13	3.17	0.11	0.93	−97.47	
5	Msat + mtDNA	206.44	6.49	0.02	0.96	−97.22	
6	Geo + Msat	206.45	6.49	0.02	0.98	−97.23	
7	Geo + mtDNA	206.46	6.5	0.02	1	−97.23	
8	Geo + Msat +mtDNA	211	11.04	0	1	−97.17	
(b)							
1	Intercept-only	198.06	0	0.55	0.55	−96.53	$\frac{Model\ 1}{Model\ 2}=3.58$
2	mtDNA	200.6	2.55	0.15	0.71	−96.21	
3	Geo	201.23	3.18	0.11	0.82	−96.53	$\frac{Model\ 2}{Model\ 3}=1.37$
4	Msat	201.24	3.18	0.11	0.93	−96.53	
5	Msat + mtDNA	204.32	6.26	0.02	0.96	−96.16	
6	Geo + mtDNA	204.4	6.35	0.02	0.98	−96.2	
7	Geo + Msat	205.04	6.99	0.02	1	−96.52	
8	Geo + Msat +mtDNA	208.98	10.92	0	1	−96.16	

better than any model including a predictor variable. For back display, the best-fit model suggests an inverse relationship between probability of back display and mtDNA distance. The evidence ratio for this model was only 1.8–2.6 times better than three other models that had AICc values ≤2.0.

4 | DISCUSSION

Our study provides evidence that both male and female red-eyed treefrogs exhibit unique, population-specific reproductive behavior. Male advertisement calls differed in mean dominant frequency
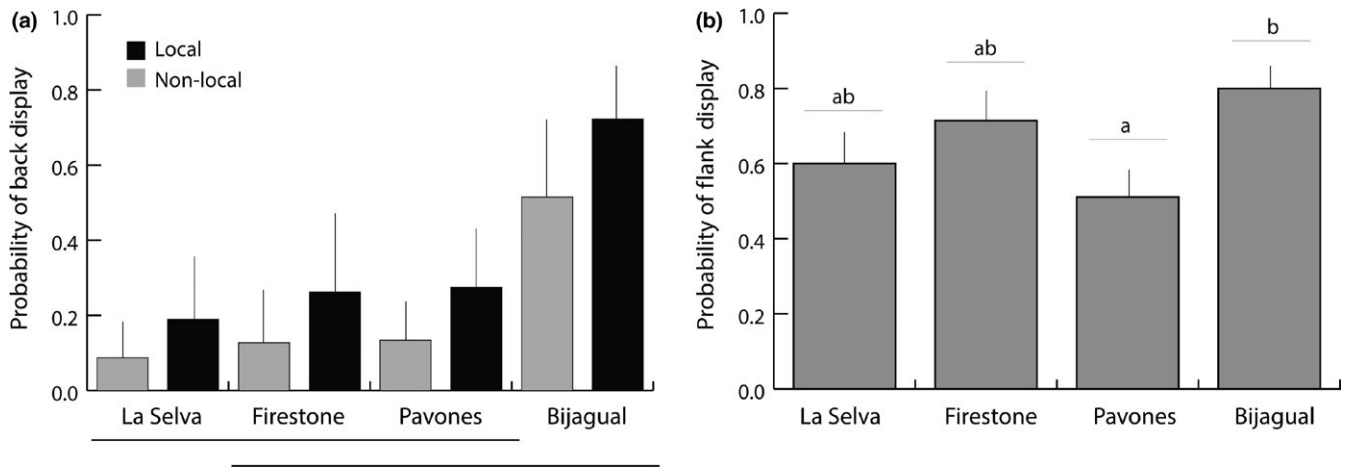
FIGURE 3 Female mating displays varied across four populations. Probability of back display (a) and flank display (b) differed across populations (shown are means ± *SE*). Females were more likely to back display to local male stimuli than to non-local stimuli (ranging from 1.4-fold more likely at Bijagual to 2.2-fold at La Selva; best-fit log-linear model), but females displayed flanks equally to local and non-local male stimuli. Thus, trials with local and non-local males were pooled together for flank display. Higher probabilities of flank display compared to back display are likely explained by the functions of these displays (see discussion) and are not a result from pooling data. For back display (a), underlined populations denote non-significant pairwise comparisons (Tukey's contrasts, *p* < .05), that is, La Selva and Bijagual show population-level differences in female response behavior regardless of male stimulus. For flank display (b), treatments represented by different lower-case letters denote significant pairwise comparisons (Tukey's contrasts, *p* < .05)

TABLE 4 Geographic and genetic distances between populations fail to predict variation in female response behavior. Values calculated for the model selection for logistic regression analysis using three continuous predictor variables: geographic distance (km) and two measures of genetic distance (F_{ST} based on microsatellite and mtDNA markers) and two categorical response variables: flank display (a) and back display (b). Geographic and genetic distances were weak predictors of the occurrence of back and flank displays

Model	Predictor variables	AICc	ΔAICc	AICc weight	Cumulative weight	Log-likelihood	Evidence ratio
(a)							
1	Intercept-only	252.43	0	0.35	0.35	−124.19	$\frac{Model\ 1}{Model\ 2} = 2.12$
2	Geo	253.93	1.5	0.17	0.52	−123.9	
3	Msat	254.09	1.65	0.15	0.67	−123.98	$\frac{Model\ 2}{Model\ 3} = 1.08$
4	mtDNA	254.32	1.88	0.14	0.81	−124.1	
5	Geo + Msat	255.95	3.52	0.06	0.87	−123.87	
6	Geo + mtDNA	256.01	3.58	0.06	0.92	−123.9	
7	Msat + mtDNA	256.17	3.74	0.05	0.98	−123.98	
8	Geo + Msat + mtDNA	258.03	5.59	0.02	1	−123.85	
(b)							
1	mtDNA	219.5	0	0.31	0.31	−106.69	$\frac{Model\ 1}{Model\ 2} = 1.79$
2	Msat	220.66	1.16	0.17	0.48	−107.27	
3	Msat + mtDNA	221.38	1.88	0.12	0.6	−106.58	$\frac{Model\ 2}{Model\ 3} = 2.55$
4	Geo + mtDNA	221.44	1.94	0.12	0.72	−106.61	
5	Intercept-only	221.8	2.3	0.1	0.82	−108.87	$\frac{Model\ 3}{Model\ 4} = 1.43$
6	Geo	222.46	2.96	0.07	0.89	−108.17	
7	Geo + Msat	222.73	3.23	0.06	0.95	−107.26	
8	Geo + Msat + mtDNA	223.23	3.73	0.05	1	−106.45	

(Hz) and bandwidth (Hz), following the general trend that populations on each versant are more similar to each other than are allopatric populations. Despite similarities among neighboring populations, we were able to predict natal populations based on these two call parameters. We also demonstrate geographic variation in female responses: females across populations all use back

and flank displays, but populations differed in frequency and duration of displays. Moreover, the frequency of flank display in each population was similar regardless of whether females were presented with local or non-local stimuli, indicating population-specific behavior rather than context-specific behavior. In contrast, back displays were more frequently observed in the presence of a local male stimulus. Female population membership can also be predicted based on female response behavior, although this classification was weaker than male classification (Table 2). Further, neither population variation in male advertisement calls nor female behavior was well explained by geographic or genetic distance, indicating a role for localized selection and/or drift. Overall, our study is the first to demonstrate geographic variation in female courtship responses in a frog.

Reproductive isolation should be accelerated when divergence in male courtship signals is coupled with divergence in female response to those signals (Edward & Chapman, 2011). Our study suggests that these criteria might be met for red-eyed treefrogs: Females not only show local mate choice for population-specific male courtship signals, but also exhibit population-specific courtship behaviors themselves. For example, females were more likely to perform a back display to local male stimuli in our mate choice trials. It is possible that differences in male courtship signals among populations mediate the evolution of variation in female behavior, a phenomenon previously demonstrated to underlie reproductive isolation in birds (Coleman, Patricelli, & Borgia, 2004) and fishes (Gonzalez-Zuarth, Vallarino, & Garcia, 2011). Thus, coevolution between male courtship and female responsiveness may lead to local variants (i.e., population-specific courtship repertoires), which can affect mate recognition among divergent populations and promote assortative mating. This in turn can accelerate sexual isolation and promote speciation.

Male courtship signals show stronger population and regional differentiation than female responses to those displays. One possible explanation is that differences in selection pressure arise due to signal function. Male calls function for both intra- and intersexual communication (Ryan, 2001; Wells, 2007), while female visual signals appear to be only directed toward males. Female response behavior typically consists of relatively subtle signals and cues, such as posture or the distance the female keeps from the male, and may convey information to the male about her interest in him as a potential mate. Males may use this information to maximize their chances for successful courtship (West & King, 1988; Balsby & Dabelsteen, 2002; Meffert & Regan, 2002; Santangelo, 2005). Another possible explanation is that ecological selection contributes, in part, to the evolution of male advertisement signals (Ryan, Tuttle, & Rand, 1982; Maan & Cummings, 2008). Heterogeneity of selective forces, in the form of predation, competition, and/or environmental pressures, can lead to the evolution of behavioral variants (Zuk & Kolluru, 1998; Foster & Endler, 1999; Bernal, Rand, & Ryan, 2006; Akre, Farris, Lea, Page, & Ryan, 2011).

The use of visual signaling in anurans is known from both diurnal and nocturnal frogs (Haddad & Giaretta, 1999; Amézquita & Hödl,

2004). High visual sensitivity in anurans allows for visual communication even in low-light conditions (Aho, Donner, Helenius, Larsen, & Reuter, 1993; Yovanovich et al., 2017); in fact, many nocturnal anuran species employ visual mating displays such as arm waving, foot flagging, and vocal sac inflation (Halloy & Espinoza, 2000; Gomez et al., 2009; Toledo & Haddad, 2009; de Sa, Zina, & Haddad, 2016). Our behavioral observations support this conclusion: Female response signals (flank and back displays) are performed only in the presence of males, suggesting its role for intersexual communication. Further, our observations, both in the field and during mating trials, reveal that flank and back displays are performed sequentially and thus could provide clues as to the function of each display. Back displays occur last in the mating sequence, typically resulting in amplexus (Pyburn, 1970), and therefore might be an indication of female choice. Flank displays, on the other hand, could be viewed as a signal of interest and occur earlier in the mating sequence, during the period in which a female is approaching and assessing an advertising male. If this is true, it suggests that flank and back displays differ in function and could therefore elicit varying responses from males. In addition, the roles of other forces potentially shaping female behavior (e.g., variation in male signal transmissibility, population sizes and dynamics, or interactions of male and female behavior among populations) remain to be tested.

Recent studies focusing on courtship dynamics have acknowledged the interactive nature of the behaviors that precede mate choice (Medina, Garcia, Urbina, Manjarrez, & Moyaho, 2013; Yoshikawa, Ohkubo, Karino, & Hasegawa, 2016). In a mutual mate choice scenario, both males and females exhibit courtship displays to advertise individual quality (Tobias, Viswanathan, & Kelley, 1998; Heinig et al., 2014); thus, partner assessment is a reciprocal process, such that both sexes process information on the quality of a mate. This can occur even in scenarios of unequal investment between the sexes, as in many frogs where females invest more in reproduction and are therefore thought of as the choosy sex. Our finding that female mating displays differ among populations such that males could play a role as selective signal receivers could thus represent a paradigm shift in female choice-based anuran mating systems. Indeed, previous mate choice trials using live males and females suggest that males discriminate among potential mates; some males that were approached by a female (back display) did not engage in amplexus (Jacobs et al., 2016). Future studies need to explore the consequences of female response behavior and the role of male mate choice in red-eyed treefrogs. Our work sheds light on the complexity and sex specificity of mating signal evolution and can be applied broadly to many sexually reproducing taxa: Courtship signals and the responses they elicit are elaborate, nuanced, and comprised of geographic behavioral variants.

frog models. A.D. Robertson developed robotic models. D.A. Gray, R.E. Espinoza, L.R. Forti, and members of the Robertson lab provided feedback that substantially improved this manuscript.

COMPETING INTERESTS

We have no competing interests.

ETHICS

This study was conducted in accordance with the current laws in Costa Rica and with approval of the Sistema Nacional de Áreas de Conservación of the Ministerio de Ambiente y Energía (SINAC-SE-GASP-PI-R-086-2014). All procedures were approved by the California State University, Northridge Institutional Animal Care and Use Committee (1213-012a).

DATA ACCESSIBILITY

Behavior and call data: Figshare (https://doi.org/10.6084/m9.figshare.3581481.v1).

AUTHORS' CONTRIBUTIONS

JMR, KK, MA, AV, NGS, and CYO designed the study. MA, AV, NGS, and CYO carried out the experiment. SRD, KK, and MA conducted statistical analyses. All authors contributed to writing the manuscript and have given final approval for publication.

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