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Original Article

Female mate preferences do not predict male sexual signals across populations

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New species can arise when female preferences and male sexual signals diverge across populations and thereby reduce mating between populations. Under this hypothesized mechanism for speciation, mate preferences and sexual signals should be correlated, but divergent, across populations. We evaluated this prediction using spadefoot toads (*Spea multiplicata*). We measured a sexually selected male signal (call rate) for which female preferences are known to vary across populations in response to the risk of hybridizing with another species. Contrary to expectation, we found no correlation between male call rate and female preferences across populations. We discuss possible mechanisms of this pattern, including the effect of gene flow from heterospecifics on male call rate. Our results suggest that, even when populations vary in mating traits, the independent evolution of female preferences and male sexual signals might impede reproductive isolation between populations.

Key words: **hybridization, introgression, reinforcement, reproductive isolation, sexual selection, speciation.**

INTRODUCTION

Evolutionary biologists have long sought to understand how new species arise. One proposed mechanism of speciation is that mating traits diverge between populations and this reduces mating—and concomitantly gene flow—between them, thereby promoting speciation (Coyne and Orr 2004; Ritchie 2007). Understanding how and when mating traits diverge is therefore an important component of understanding the speciation process (Coyne and Orr 2004; Price 2008).

Mating traits can diverge between populations whenever they are subject to differences in the strength, direction, or targets of sexual selection across environments (Arnqvist 1992; Gerhardt 1994; Carroll and Salamon 1995; Boughman 2001; Gabor and Ryan 2001; Kwiatkowski and Sullivan 2002; Rundle et al. 2005; Jang and Gerhardt 2006; Seehausen et al. 2008; Pfennig and Rice 2014). Yet, whether divergence in mating traits actually reduces gene flow likely depends on the extent to which divergence occurs in the traits possessed by *both* sexes (reviewed in Pfennig 2016; Calabrese and Pfennig 2020). In particular, if mate preferences diverge between populations, but sexual signals do not, then populations are unlikely to diverge if choosy individuals cannot discriminate between prospective mates of their own population versus those from alternative populations (Lemmon 2009; Moran and Fuller 2018). Indeed, populations are expected to diverge genetically only when mate preferences and sexual signals diverge concomitantly (Kirkpatrick 1982; Spencer et al. 1986; Liou and Price 1994; Kirkpatrick and Servedio 1999; Servedio 2000; McPeek and Gavrilets 2006).

Mate preferences and sexual signals are generally expected to coevolve (Fisher 1930; Lande 1981; Andersson 1994), but this need not be the case. In particular, mate preferences and sexual signals can be subject to different patterns of gene flow, drift, or selection. Consequently, mate preferences and sexual signals might not co-vary across different populations (Wheatcroft and Qvarnström 2017). Despite this possibility, relatively few studies have evaluated whether such traits diverge in concert as expected (but see, e.g., Houde and Endler 1990; Boughman 2001; Höbel and Gerhardt 2003; Hoskin et al. 2005; Rundle et al. 2005; Lemmon 2009). Nevertheless, evaluating divergence in both preferences and signals is a critical prelude to understanding whether reproductive isolation among populations arises from divergence in mating traits.

A particularly powerful context in which to evaluate whether and how mating traits diverge between populations is in systems where individuals of a given species co-occur and risk costly reproductive interactions with another species in some populations (i.e., sympatry) but not others (i.e., allopatry). In particular, in sympatric populations, selection will favor the evolution of mating traits that minimize costly mating interactions with heterospecifics (a process known as reproductive character displacement; Pfennig and Pfennig 2009; Pfennig and Pfennig 2012). However, because such selection does not occur in allopatry, mating traits that evolve

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in sympatry also diverge from those in allopatric populations (reviewed in Pfennig and Pfennig 2012). A downstream consequence of this divergence could be speciation between conspecific populations that are sympatric versus allopatric with other species if they become so divergent in mating behaviors that individuals from the different population types do not mate with each other (Ortiz-Barrientos et al. 2009; Pfennig and Pfennig 2009, 2012; Abbott et al. 2013).

Yet, the extent to which sympatric and allopatric populations diverge concurrently in mate preferences and sexual signals could depend on both gene flow or selective factors that differentially impact males and females (Pfennig 2016; Calabrese and Pfennig 2020). Regarding the former, sex-biased gene flow could cause differences in the extent of character displacement in mate preferences versus sexual traits (Pfennig 2016; Calabrese and Pfennig 2020). Likewise, gene flow between hybridizing species in sympatry can generate differences in the extent of character displacement in preferences versus sexual signals if male and female traits are differentially impacted by introgression (Perrot-Minnot et al. 2004; Kulmuni and Pamilo 2014; Runemark et al. 2018).

Moreover, mate preferences and sexual signals might experience different selective patterns beyond what arises in the unfolding of character displacement. For example, other biotic factors aside from reproductive interactions with heterospecifics (e.g., predation) or abiotic factors might limit how sexual signals respond evolutionarily to mate preferences (Andersson 1994; Endler and Basolo 1998). Such limits might break down correspondence between mate preferences and sexual signals across populations. Thus, although divergence in mating traits between sympatric and allopatric populations is expected to be likely (Pfennig and Pfennig 2009, 2012; Abbott et al. 2013; Calabrese and Pfennig 2020) and the possibility that such divergence can drive reproductive isolation among conspecific populations has received some support (Pfennig and Pfennig 2012; Abbott et al. 2013; Pfennig 2016), additional work is needed that examines whether mate preferences and sexual signals diverge in tandem as expected (Price 1998; Ritchie 2007; Pfennig 2016; Calabrese and Pfennig 2020).

To address this issue, we examined variation in female preferences and male signals among populations of Mexican spadefoot toads (*Spea multiplicata*) that vary in the risk of mating with a congener, *S. bombifrons*. Female mate preferences in *S. multiplicata* have diverged between sympatry and allopatry (Pfennig 2000; Pfennig and Rice 2014), but whether male sexual signals have co-evolved with preferences in these populations is unknown. In particular, female *S. multiplicata* in allopatric populations prefer males with faster call rates (an indicator of mate quality: Pfennig 2000, 2008; Kelly et al. 2019), whereas deleterious hybridization in sympatry has led to increased preference for slow-calling males (whose calls are more displaced from *S. bombifrons*). We predicted that male call rates would be faster in allopatry than in sympatry, in response to selection by female choice in those population types. Moreover, because females are subject to a mate quality-species identity tradeoff when evaluating male call rates (see "Study system" below), variation in hybridization risk or other factors that impact the fitness outcomes of mate preferences may vary within as well as between sympatry and allopatry. Therefore, we further predicted that female mate preferences would predict male call rates across populations. We did not find support for either prediction, suggesting that male signals and female mate preferences do not necessarily co-evolve—or diverge—as is often suggested under models of speciation.

METHODS

Study system

Spea multiplicata occurs in the southwestern United States and Mexico. Mating takes place in ephemeral breeding ponds where males aggregate and call to attract females. Females initiate amplexus by closely approaching or touching a male. In the desert regions, spadefoots breed on the first night after a pond fills; females do not mate multiply at a breeding aggregation nor do they mate multiply in a season. It is common to observe many unmated males (but not females) at the end of breeding aggregations (Sullivan and Sullivan 1985; personal observation); as such, sexual selection on male calls by female choice is expected to be strong.

Female *S. multiplicata* choose males on the basis of male call rate (Pfennig 2000; Pfennig and Rice 2014). In populations where *S. multiplicata* is the only *Spea* species (allopatry), females prefer faster-calling males that are in better condition (Pfennig 2008), have higher fertilization success (Pfennig 2000), and sire tadpoles that have greater adaptive plasticity (Kelly et al. 2019) and higher fitness (Pfennig 2008; Kelly et al. 2019). However, in some populations, *S. multiplicata* co-occur with a congener, *S. bombifrons*, with which mating is deleterious to *S. multiplicata* females (Pfennig and Simovich 2002; Wünsch and Pfennig 2013; see also Seidl et al. 2019). In these sympatric populations *S. multiplicata* females are less likely to prefer fast calls than are females in allopatry (Pfennig 2000; Pfennig and Rice 2014). Because *S. bombifrons* and hybrid males call faster (on average around 70 calls/min and 50 calls/min, respectively) than *S. multiplicata* (on average around 31 calls/min), females in sympatry presumably prefer slower call rates to reduce their risk of hybridizing. However, by mating with slower calling conspecifics, they forgo the fitness benefits of mating with high-quality (fast-calling) males (Pfennig 2000, 2008; Kelly et al. 2019).

Although *S. bombifrons* and *S. multiplicata* calls differ in a number of call features, the available evidence suggests that *S. multiplicata* females discriminate between species using primarily call rate (Pfennig 2000; Chen and Pfennig 2020), which is also the call feature they use to assess conspecific quality (Pfennig 2000). Because this tradeoff between avoiding hybridization and choosing high-quality mates has led to variation in female preferences for call rates (Pfennig 2000; Pfennig and Rice 2014), sampling populations across sympatry and allopatry is crucial to evaluating whether female preferences and male signals co-evolve in this system.

Male call rate

We recorded calls from *S. multiplicata* males at breeding aggregations in the San Simon valley of Arizona and New Mexico, USA, between 1996 and 2018. Recordings spanned 21 populations that are sympatric (i.e., *S. bombifrons* and/or hybrid males have been observed at the breeding aggregation) and allopatric (where only *S. multiplicata* have been observed). Distances between populations in the study ranged 0.54–35.4 km, with an average pairwise distance of 13.6 km. Spadefoots have limited dispersal and significant genetic differentiation has been observed between populations breeding as little as 0.4 km apart (Pfennig and Rice 2014). Some populations were sampled in multiple years, for a total of 35 unique breeding aggregations that were sampled. We sampled breeding aggregations opportunistically, because the timing of breeding is unpredictable and depends on rainfall patterns. At each breeding aggregation, we recorded as many males as possible given the size of the aggregation and logistical constraints. We sampled males arbitrarily and moved throughout the pond to ensure sampling throughout the aggregation. In 20 of the 35 aggregations, we collected each male after recording him (if possible; some escaped capture) and took a tissue sample for subsequent genetic analyses. We also measured the water temperature of the breeding pond at the time of recording.

Hybridization occurs naturally in the sympatric populations (Simovich 1985, 1994; Pfennig and Simovich 2002), and although F_1 hybrids can be distinguished by call features, backcross hybrids can have similar calls to *S. multiplicata*. We excluded F_1 hybrids identifiable by call phenotype, and used genetic data to further screen for early-generation hybrids and exclude them from our analysis. We genotyped those males for which we had tissue samples using species-specific restriction-fragment length polymorphisms at 9 loci following (Pfennig et al. 2012). Briefly, we amplified DNA at each locus using PCR, digested the PCR product with restriction enzymes, and visualized DNA fragment length using gel electrophoresis. Each locus was scored with a 1, if homozygous for the *S. bombifrons* allele, a 5, if homozygous for the *S. multiplicata* allele, or a 3 if heterozygous. Scores were averaged across loci for each individual, resulting in a “genotype score” ranging from 1 (pure *S. bombifrons*) to 5 (pure *S. multiplicata*). Males with genotype scores of less than 4 were excluded from the analysis, thereby excluding early-generation hybrids. We excluded 11 such males on the basis of genotype score.

Genotype data were available for 228 of the 526 males included in the analysis (43%); for sympatric males specifically, 154 out of 242 (64%) were genotyped, and 74 out of 284 (26%) of the allopatric males were genotyped. Our median genotype score was 4.82 (the genotype score of a male with only one *S. bombifrons* allele across all markers is 4.78). Males with genotype scores between 4 and 5 have between one and four *S. bombifrons* alleles out of 18 possible, regardless of their distribution across the 9 loci. For example, a male that is homozygous for the *S. bombifrons* allele at one locus and homozygous for the *S. multiplicata* alleles at all the 8 remaining loci would have the same genotype score (4.56) as a male that is heterozygous at two loci and homozygous *S. multiplicata* at the remaining 7 loci. There was no difference in call rates between pure *S. multiplicata* males (i.e., genotype score = 5; mean = 35.43 ± 0.36 calls/min) and those with *S. bombifrons* alleles (i.e., $4 \leq$ genotype score < 5; mean = 35.21 ± 0.38 calls/min; $t = 0.42$, $df = 225.7$, $p = 0.67$). The subset of call data from populations for which female preference data were also available consisted of 419 male calls from 14 populations. Genotype data were available for 226 of these males, or 54% of this subset, with median genotype score = 4.82.

We digitized call recordings and analyzed them using Audacity(R) v 2.1.3 (Audacity Team 2020). For each male, we selected one to three call series (periods of continuous calling) for analysis, containing up to 12 total calls. Series were selected to maximize recording quality of the analyzed calls (e.g., minimize wind noise). We measured call rate as the number of calls contained in the series divided by the duration of the series. For males with multiple call series analyzed, the call rate was averaged across the series.

Female preference

We tested preferences of 169 females from 14 populations for fast versus slow calls. The populations sampled, and the sample size, were determined by logistical constraints: we tested all *S. multiplicata* females from the San Simon Valley that were available in our lab colony at UNC and gravid (therefore receptive for mating) during the testing period of 2015 to 2020, from populations for which we also had male call data. Animals in the lab colony had been collected as adults from natural populations and returned to UNC-Chapel Hill. There they were maintained on a reverse 14-h light:10-h dark cycle and fed nutrient-dusted crickets ad libitum. Sample size per population ranged from 4 to 25 females (with a mean of 12 and a median of 10.5 females per population).

We assessed preferences for call rate using two-choice phonotaxis tests, following previous methods (Pfennig 2000; Pfennig and Rice 2014). We presented females with a choice of synthetic *S. multiplicata* calls presented at a fast call rate (37 calls/min) versus a slow call rate (26 calls/min). The calls were produced using Audacity software. We generated a sine wave of 1337 Hz for 0.2 s. and used the fade out function to create a triangular pulse. The pulse was repeated (with interspersed silent intervals to generate a pulse rate of 24.2 pulses/s) to create a call of 0.95 s duration. This synthetic call was then repeated (with silent intercall intervals of either 1.412 s or 0.690 s to create either the slow or the fast call rate stimulus, respectively).

These call rates are within the observed range of call rate variation in the wild (see results and figures in this article). Other call characteristics (e.g., pulse rate, call duration) were set to species-typical values (Pfennig 2000). Calls were broadcast from two Sony SS-B1000 speakers placed on platforms on opposite sides of a circular water-filled pool 1.8 m in diameter. Two platforms without speakers were placed 90° from the speaker platforms to allow females to rest out of the water without approaching a speaker. Each speaker played one call stimulus (either the fast or slow call) for the duration of the trial, and stimuli were played in alternating 30-s bouts. The speaker from which the fast call rate was broadcast was switched between trials, and the leading stimulus was randomized.

We began each trial by placing a female onto a central platform in the center of the arena to acclimate for 5 min under an opaque container. The calls began playing at the start of the acclimation interval. After acclimation, the female was released to move around the pool. We ended the trial after 30 min or when the female made a choice, whichever came first. We defined a choice as the point at which a female touched the platform supporting the speaker. Females initiate amplexus in nature by closely approaching or touching males, so this choice criterion is biologically relevant. Females that did not make a choice were considered unresponsive and excluded from subsequent analyses. For each population, we calculated the proportion of females choosing the fast call stimulus as the number that chose the fast call rate stimulus divided by the total number of females that chose either of the stimuli.

Statistical analysis

We used R version 3.6.1 (R Core Team 2019) for all analyses. To ascertain if females in either sympatry or allopatry had preferences for call rate that differed from a random 50:50 expectation, we used exact binomial tests. We also tested for an effect of year of testing and the elapsed time since females were collected in the wild on female preferences using binomial mixed effects models. To do so, we modeled mate preference as the response variable, with either year of testing (as a factor) or time in the colony in years as the fixed effect, and population as a random intercept, using the glmer function (package lme4: Bates et al. 2015). We then compared these models to a null model with only the random intercept of population, using a likelihood ratio test to assess significance. We assessed whether sample size of females per population predicted female preferences in two ways. First, we modeled binomial outcomes of choice tests as a function of sample size in each population using glm (package stats: R Core Team 2019) and tested the significance of sample size via a likelihood ratio test. Second, we calculated a non-parametric spearman's correlation coefficient for the relationship between per-population sample size and proportion of females choosing the fast call rate stimulus in each population.

The unit of observation for our analysis of male calls was the individual male. Because call rates are temperature-dependent in *Spea*, we first calculated temperature-corrected values for individual males' call rates by standardizing to a common temperature of 20.4° C. To do so, we fit a linear model of the effect of water temperature on call rate of all males included in the analysis, then added the residuals of this model to each male's predicted call rate at 20.4° C to obtain a temperature-corrected call rate for each of the 526 males in the dataset.

We first tested the effects of elevation, population type (sympatry vs. allopatry), and individual males' genotype score on the broader dataset of male call rates (i.e., including populations for which we do not have female preference data) using linear mixed-effects models (package lme4: Bates et al. 2015). Elevation and population type were not included in the same models to avoid multicollinearity. For all models, year of recording was included as a fixed effect to control for changes in call rates over time (variation in call rate across years is linear: Calabrese and Pfennig, manuscript in review; see also Supplemental Methods and Results), and population was included as a random intercept to account for the non-independence of males recorded from the same population. We used the lmerTest package (Kuznetsova et al. 2017) to assess statistical significance of predictor variables in linear mixed models via ANOVA, with Kenward–Roger method for computing denominator degrees of freedom.

We then used linear mixed-effects models to test whether female preferences predicted male call rates across those populations for which we had both kinds of data. We used temperature-corrected call rates as our response variable, and the log odds of females choosing fast calls in each population (i.e., logit of proportion preferring fast) as a fixed-effect predictor variable. Elevation and year of recording were also included as fixed effects. Population was included as a random intercept to account for the non-independence of males recorded from the same population. We weighted the observations by number of females sampled per population in preference tests (using the "weights" argument in the lmer function), in order to incorporate uncertainty in female preferences due to sampling effort. We repeated these analyses on the subset of allopatric populations (see Supplementary Methods and Results).

Figures were constructed using ggplot2 (Wickham 2016), ggpubr (Kassambara 2019), ggbeeswarm (Clarke and Sherrill-Mix 2017), sjPlot (Lüdecke 2019), and cairo (Urbanek and Horner 2019). All animal care and procedures were approved by the IACUC committee at the University of North Carolina, Chapel Hill (current protocol 17-073.0-C).

RESULTS

Females from allopatric populations significantly preferred fast calls (exact binomial test: 70 chose fast, 40 chose slow, probability of preferring fast = 0.636, $p = 0.005$). By contrast, *as a group*, females from sympatric populations did not display a significant preference for either call rate stimulus (30 chose fast, 29 chose slow, probability of choosing fast = 0.508, $p = 1$). This result is consistent with past work showing significant preferences for fast calls in allopatry but not sympatry, although previous work found a significant preference for the slower call rate stimulus in sympatric females (Pfennig 2000; Pfennig and Rice 2014).

Female preferences can differ between allopatry and sympatry for reasons other than overlap with heterospecifics, but we found no evidence supporting this possibility. We found no effect of either the year of testing or the length of time females were held in the colony on female preferences (Supplementary Table S1). Thus, female age or time in captivity did not impact their mate preferences. Moreover, sample size of females per population did not predict choices in each population (LRT: $p = 0.4$), nor did it correlate with proportion of females in each population that chose the fast call rate stimulus (spearman's $r = 0.12$, $p = 0.7$). Thus, the patterns of female preference we observe cannot be attributed to uneven sampling across populations.

We analyzed a total of 526 *S. multiplicata* males' calls, from 12 allopatric and 9 sympatric populations with the faster calling *S. bombifrons*. Contrary to expectation, male calls in sympatric populations tended to be faster than those in allopatric populations, although the difference between them was not statistically significant at an alpha level of 0.05 (Table 1; Figure 2; slope = 1.73 ± 0.96, $F = 3.2$, $p = 0.09$). Because the presence of *S. bombifrons* varies with elevation, we examined whether elevation predicts male call rate and found that male call rate *decreased* with *increasing* elevation (Table 1; slope = −0.01 ± 0.005, $F = 8.7$, $p = 0.009$, Figure 1). Thus, *S. multiplicata* male call rate is slower at higher elevations (where *S. bombifrons* is absent: Simovich 1985; Pfennig and Pfennig 2005) and faster at lower elevations that include sympatry. This pattern is unexpected given the above results that females from sympatry are more likely than are females from allopatry to prefer slower call rates in the presence of the faster calling *S. bombifrons* (see also Pfennig 2000; Pfennig and Rice 2014).

For those males in the analysis that were genotyped, genotype did not predict call rate (Supplementary Table S2). When we compared the call rate distribution of males that were genotyped to the call rate distribution of all males included in the analysis, we found that excluding ungenotyped males under-sampled the low end of the call rate distribution (Supplementary Figure S1) and under-sampled

Table 1

Temperature-corrected call rates of $N = 526$ males from 21 populations modeled as a function of elevation (1132–1513 m), year of call recording (1996–2018), and population type (sympatry or allopatry, with allopatry as the reference population type)

Model	Parameter	Estimate	SE	*F*	*p*
Call rate ~ elevation + year + (1 | population)	Elevation	−0.01	0.005	8.7	0.009
	Year	0.13	0.03	15.7	<0.0001
Call rate ~ population type + year + (1 | population)	Population type	1.73	0.96	3.2	0.09
	Year	0.14	0.03	16.1	<0.0001

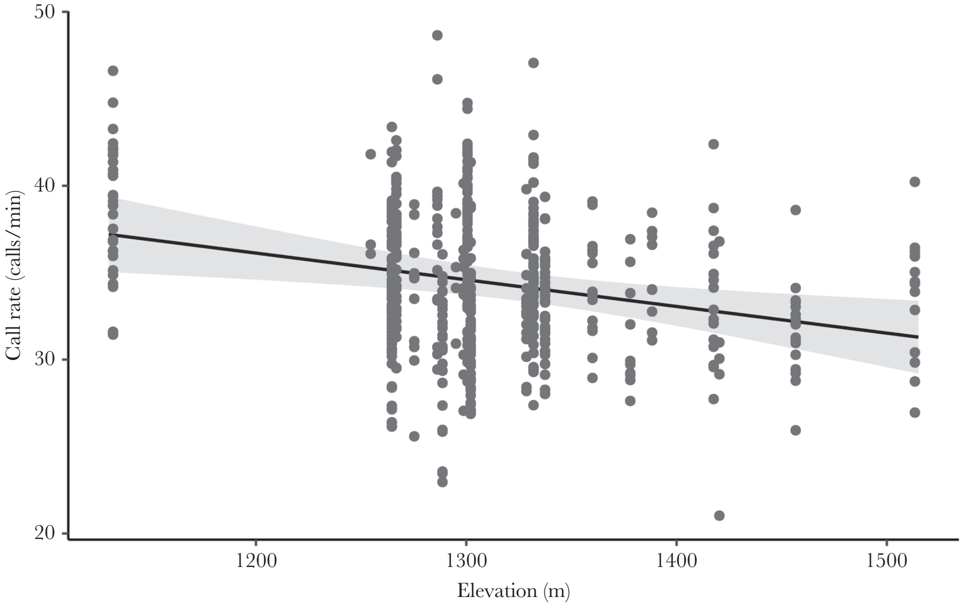

Figure 1
Marginal plot of call rates across elevation for each of 526 males from 21 populations. The slope of the effect of elevation on call rate is indicated by the line, and is modeled as a fixed effect, together with year of recording as a fixed effect and with population as a random intercept. Shading shows 95% confidence interval. Elevation significantly predicts call rate ($p = 0.009$; Table 1).

allopatry (allopatric males were 54% of the total analyzed dataset but only 32% of genotyped males). Because hybrids have faster call rates than *S. multiplicata* and do not occur in allopatry, these results suggest that ungenotyped males were not unidentified early-generation hybrids.

We found no association between female preference for call rates in a population and male call rates across populations (slope = 0.46 ± 0.92, $F = 0.2$, $p = 0.64$; Table 2; Figure 3). Moreover, female preference for fast call rates in a population also did not significantly predict male call rates after accounting for the effects of elevation on call rate (Table 2). Results did not differ when we repeated these analyses using generalized additive mixed-models to account for non-linear variation across years (see Supplemental Methods and Results, Supplementary Figures S3–S5) or using only the subset of populations that are allopatric with *S. bombifrons* (Supplementary Tables S6–S7). Thus, female mate preferences do not predict male sexual signals across these populations.

DISCUSSION

We examined whether female mate preferences predict male sexual signals across populations that differed in their interactions with another species, a common selective agent on mating traits (Servedio and Noor 2003; Coyne and Orr 2004; Price 2008; Ortiz-Barrientos et al. 2009; Pfennig and Pfennig 2012; Comeault and Matute 2016; Pfennig 2016). We found that, although allopatric females prefer faster call rates and sympatric females do not prefer faster call rates (Pfennig 2000; Pfennig and Rice 2014), allopatric males tend to call more *slowly* than sympatric males. This pattern is opposite of what is expected if male signals evolve in response to female preferences. Indeed, we found no relationship between female preference for call rate and male call rates across populations (Table 2; Figure 3).

Theory (McPeek and Gavrilets 2006; Pfennig and Ryan 2006) and evidence from other systems (Hoskin et al. 2005; Lemmon 2009; Bímová et al. 2011) suggest that, when female preferences diverge between populations that differ in the presence of heterospecifics, male signals also diverge as they co-evolve with female preferences. Our findings suggest that this will not necessarily be the case, and several explanations can account for such a result. One such explanation is the possibility that sexual selection is weaker on male sexual signals in natural populations than is expected from lab-based female preference data (Rosenthal 2017). In particular, in *S. multiplicata*, males and females mate only once at a breeding aggregation and such aggregations might occur only once in a breeding season. Although males can vastly outnumber females at aggregations, if males with the preferred call rate are limited or if females cannot afford search costs, females might mate with less preferred males rather than not mating. Any such pattern would reduce the strength of selection on calling males and result in the breakdown of an association between measured female preferences and male sexual signals across populations (because measured female preferences would not strictly determine which males mated).

Likewise, variable selection on female mate preferences might reduce the overall strength of directional sexual selection on male calls (Jennions and Petrie 1997; Cotton et al. 2006; e.g., Pfennig and Tinsley 2002). The fitness benefits of female preferences for call rate depend on the risks and costs of hybridization versus mating with a low-quality conspecific (see “Study system”). Thus, variation in *S. bombifrons* abundance could change the cost-benefit ratio of preference for slow call rate in sympatry; if such changes drive temporal changes in preferences, selection on male calls could also vary. Indeed, recent findings suggest that *S. multiplicata* females might *facultatively* modify their preferences depending on the presence of *S. bombifrons*: when *S. bombifrons* calls are played in the background of choice tests, sympatric *S. multiplicata* females significantly prefer the slower call rate stimulus (Calabrese and Pfennig, manuscript in preparation). Such plasticity highlights how female preferences can vary in response to the varying risk of hybridization. In short, female preferences and male calls could be mismatched depending on variability of selection on female preferences.

Table 2

Temperature-corrected call rates of $N = 419$ males from 14 populations modeled as a function of female preference (log odds of females choosing fast call rates in the population, as measured in phonotaxis trials), year of call recording, and elevation

Model	Parameter	Estimate	SE	*F*	*p*
Call rate ~ female preference + year + (1 | population)	Female preference	0.46	0.92	0.2	0.64
	Year	0.14	0.03	17.1	<0.0001
Call rate ~ female preference + year + elevation + (1 | population)	Female preference	1.23	0.74	2.4	0.12
	Elevation	−0.02	0.007	7.0	0.009
	Year	0.14	0.03	15.9	<0.0001

Although weak realized sexual selection on male signals is a possible explanation for why female preferences do not predict male calls in this system (Figure 3), this explanation does not account for why male calls show variation that is *opposite* that expected given female preferences (results in this paper; Pfennig 2000; Pfennig and Rice 2014). In other words, it is not clear why male calls tend to be relatively *faster* in the sympatric populations where females prefer relatively slower calls. Two possible explanations account for this pattern. First, male calls might be evolving in response to aspects of the environment other than female mate preferences. Although sexual signals should be strongly impacted by female choice, factors such as predators or the physical environment can also exert selection on sexual signals (Andersson 1994; Endler and Basolo 1998). In spadefoots, male calls have become increasingly faster for a given temperature in response to climate change, particularly at lower elevations which are more likely sympatric (Calabrese and Pfennig, in review). Why males might express faster call rates, which are more costly, despite female preferences for reduced signals is unclear and needs further investigation. Regardless, understanding how rapid environmental change, including that mediated by climate change, impacts preference-signal coevolution remains an important area of inquiry.

A second explanation for relatively faster male calls in sympatry despite weaker female preferences for fast calls in those populations is gene flow between *S. multiplicata* and *S. bombifrons*. Hybrids between the two species are viable, and hybrid females are fertile. Introgression of *S. bombifrons* alleles into *S. multiplicata* therefore occurs in sympatry (Simovich 1994; Pfennig et al. 2012; Pierce et al. 2017). Although *S. multiplicata* have evolved preferences that appear to minimize the risk of hybridization (i.e., they have undergone reinforcement: Pfennig 2000, 2003; Pfennig and Rice 2014), hybridization appears to be on-going because of mate choice by *S. bombifrons* females. Specifically, *S. bombifrons* females can benefit by hybridizing, they preferentially mate with *S. multiplicata* males when it is adaptive (Pfennig 2007; Chen and Pfennig 2020), and the frequency of hybrids in a population is higher in those populations where hybridization is adaptive (Pfennig and Simovich 2002). The call rates of *S. bombifrons* are faster than *S. multiplicata* and hybrid call rates are intermediate between the two species (Pfennig 2000), suggesting that call rate is a quantitative trait. Thus, introgression of S. *bombifrons* alleles into *S. multiplicata* could drive *S. multiplicata* call rates up in sympatry.

This explanation is consistent with our findings of faster call rates at lower elevations where hybridization risk is highest (Figure 1) and marginally faster call rates in sympatry (Figure 2). Although we found no relationship between genotype score and call rate for the males included in our study (Supplementary Table S2), it is unlikely that the small number of markers we used would provide sufficient resolution to ascertain the impact of introgression on male sexual signals. Additional work is therefore needed to evaluate this explanation for why male calls are faster than expected in sympatry given female preferences.

Regardless of why male sexual signals and female preferences are not similarly divergent between populations as expected, our results have important implications for understanding the conditions under which new species might arise. In particular, mismatches between female preferences and male calls across populations suggest that migrant males, if any, might not experience a mating disadvantage relative to residents in the population. Generally, selection against mates from alternative populations is expected to drive genetic divergence between populations that could ultimately initiate

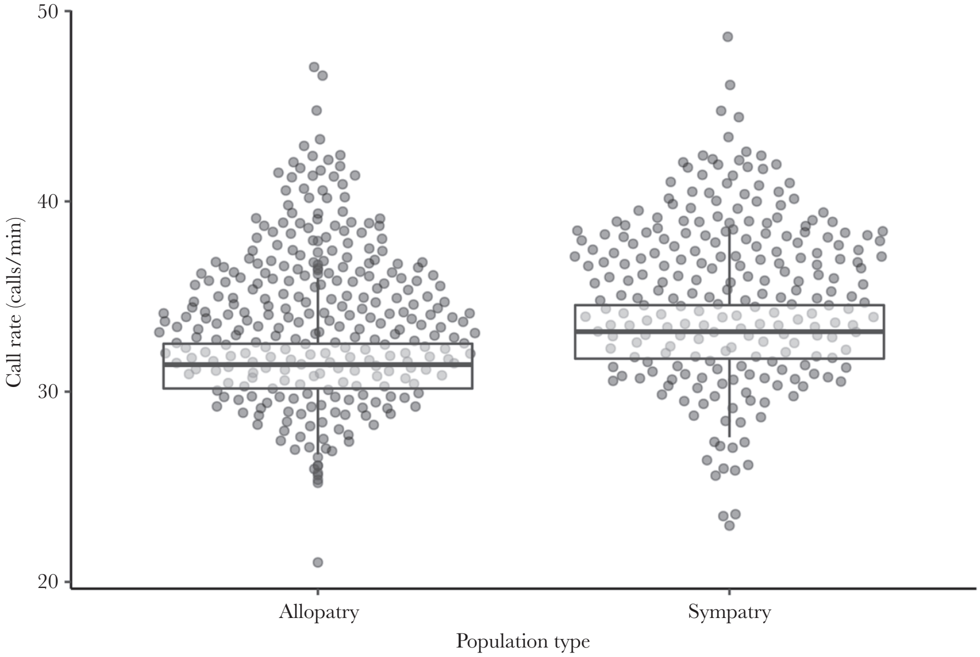

Figure 2

Temperature-corrected call rates in allopatry (N = 284 males from 12 populations) and sympatry (N = 242 males from 9 populations). Points are call rate data for individual males with horizontal jitter indicating the distribution shape (via the geom_ quasirandom function in R). Box plots are model-predicted values from 1000 bootstrap replicates (via bootMer function in R). Call rates in sympatry are marginally, but not significantly, faster than those in allopatry, when population type (sympatry/allopatry) is modeled as a fixed effect, year of recording is a fixed effect, and population is a random intercept (p = 0.09; Table 1).

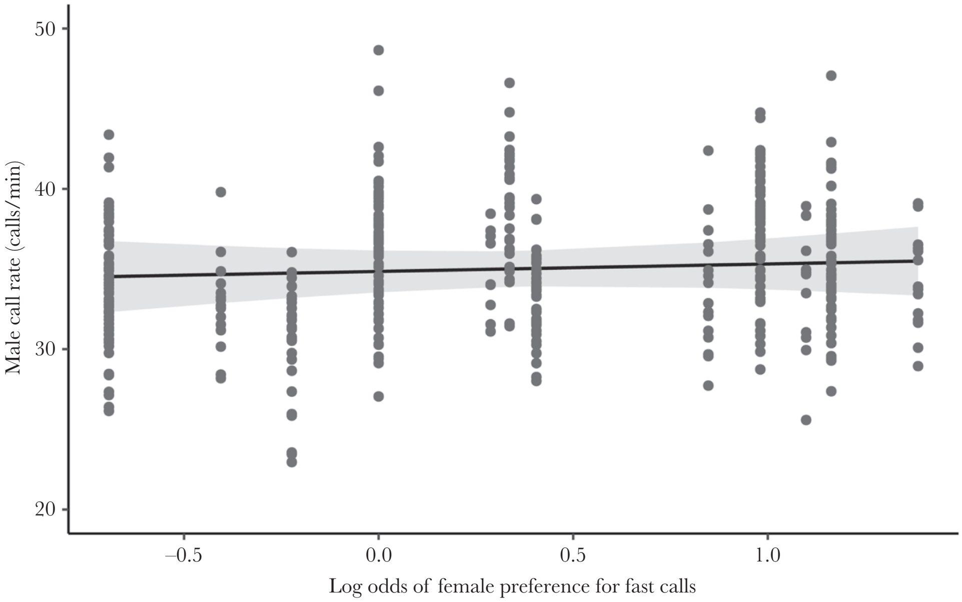

Figure 3

Call rates for N = 419 males from 14 populations for which both male call and female preference data were available, plotted against the log odds of females in the population preferring a faster call rate. Shading shows 95% confidence interval around the line showing effect of female preference (modeled as a fixed effect, with year of recording included as a fixed effect and population as a random intercept). Female preferences did not significantly predict call rates (p = 0.64; Table 2). Results were qualitatively the same if elevation was included as a fixed effect (Table 2).

speciation between them (Ortiz-Barrientos et al. 2009; Pfennig and Pfennig 2009; Pfennig et al. 2012; Abbott et al. 2013). Our results suggest that, despite divergence in female preferences between populations, males that migrate between population types might not experience a major mating disadvantage. Indeed, males migrating from sympatry to allopatry might actually be *favored* by resident females that prefer faster calling males over the slower-calling resident males (Figures 2 and 3).

If such is the case, the key issue then becomes whether, and how frequently, migration between population types occur. In *S. multiplicata*, genetic evidence suggests that sympatric and allopatric populations have diverged owing to divergent selection to avoid deleterious interactions with *S. bombifrons* (Rice et al. 2009; Pfennig and Rice 2014). These findings suggest that gene flow via migration, if any, is not countering divergent patterns of selection. Nevertheless, future work is needed to better understand whether and how mating traits impact genetic divergence between sympatric populations versus allopatric populations in this system (Pfennig 2016).

More generally, our findings suggest that female mate preferences and male sexual signals do not necessarily coevolve as expected in response to divergent selection across populations. Additional work evaluating why signals sometimes evolve in response to mate preference—and sometimes do not—remains critically important for understanding the consequences of mating trait diversity and the origins of species.

SUPPLEMENTARY MATERIAL

Supplementary data are available at *Behavioral Ecology* online.

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Data availability: Analyses reported in this article can be reproduced using the data provided by Calabrese and Pfennig (2021).

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