



Poison frog color morphs express assortative mate preferences in allopatry but not sympatry

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The concurrent divergence of mating traits and preferences is necessary for the evolution of reproductive isolation via sexual selection, and such coevolution has been demonstrated in diverse lineages. However, the extent to which assortative mate preferences are sufficient to drive reproductive isolation in nature is less clear. Natural contact zones between lineages divergent in traits and preferences provide exceptional opportunities for testing the predicted evolutionary consequences of such divergence. The strawberry poison frog (*Oophaga pumilio*) displays extreme color polymorphism in and around the young Bocas del Toro archipelago. In a transition zone between red and blue allopatric lineages, we asked whether female preferences diverged along with coloration, and whether any divergent preferences persist in a zone of sympatry. When choosing among red, blue and phenotypically intermediate males, females from monomorphic red and monomorphic blue populations both expressed assortative preferences. However, red, blue, and intermediate females from the contact zone all preferred red males, suggesting that divergent preferences may be insufficient to effect behavioral isolation. Our results highlight the complexity of behavioral isolation, and the need for studies that can reveal the circumstances under which divergent preferences do and do not contribute to speciation.

KEY WORDS: Behavioral isolation, contact zone, female preference, polymorphism, speciation.

Isolated populations of the same species can differ markedly in behavior, morphology, and physiology (Kraaijeveld et al. 2011; Miller and Svensson 2014; Brodersen et al. 2015). One potential consequence of such differentiation is a reduction in the probability that lineages will interbreed, an initial step in the process of speciation (Rundle and Nosil 2005; Kraaijeveld et al. 2011). The evolution of traits important in mate choice and acquisition may be especially likely to affect reproductive isolation (Arnqvist et al. 2000; Panhuis et al. 2001; Ritchie 2007; Kraaijeveld et al. 2011; but see Gage et al. 2002), and numerous studies have demonstrated the coevolution of mate preferences and courtship

traits (Scordato et al. 2014). While such divergence in preferences and traits is necessary for the evolution of behavioral reproductive isolation (Arnqvist et al. 2000; Panhuis et al. 2001; Ritchie 2007; Kraaijeveld et al. 2011), differentiation alone may not be sufficient to drive behavioral isolation (Dougherty and Shuker 2015; Edward 2015). When lineages are sympatric, gene flow between lineages can occur when preferences are plastic and altered by experience (Jennions and Petrie 1997), and when the costs of choosing constrain the extent to which mate preference actually determines mate choice (Irwin and Price 1999; Hebets and Vink 2007; Rodríguez et al. 2013). Preferences for hybrid phenotypes

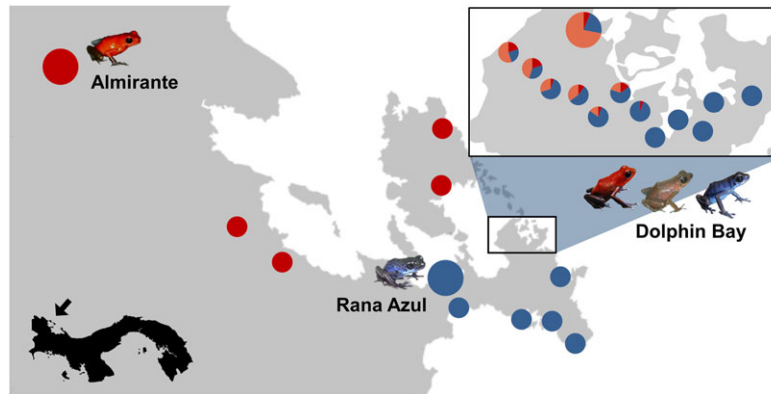


Figure 1. A transition zone between red and blue populations of *O. pumilio* in the Bocas del Toro Archipelago, Panama. Frogs used in the experiments were sampled from the three populations indicated with larger pie charts.

and/or the mating decisions of any hybrids can similarly drive gene flow between lineages (Culumber et al. 2014).

Lineages that are polytypic in sexual communication traits provide exceptional opportunities to test for the coevolution of traits and preferences and to test the hypothesis that such coevolution can drive reproductive isolation (Panhuis et al. 2001; Ritchie 2007; Twomey et al. 2016). The strawberry poison frog (*Oophaga pumilio*) is remarkably polytypic in and around the Bocas del Toro Archipelago of Panama, which reached its current conformation 1–9 kya (Gehara et al. 2013). This region is largely shaped by the rise and fall of sea-level, and hence *O. pumilio* populations have likely experienced several periods of connectivity and vicariance. This frog displays a red body with blue or black limbs throughout most of its range, but in Bocas del Toro, isolated populations display coloration spanning the visual spectrum (Summers et al. 2003; Hagemann and Pröhl 2007; Wang and Shaffer 2008; Hauswaldt et al. 2010). While minor variation may be present within islands, the most striking color variation (e.g., distinct “morphs” of different dominant color) occurs among even the most recently isolated island populations, supporting the hypothesis of rapid divergence in allopatry (Gehara et al. 2013).

As in other poison frogs (Dendrobatidae), coloration of the toxic *O. pumilio* is hypothesized to function as an aposematic signal (Darst et al. 2006; Saporito et al. 2007), but may also serve in intersexual communication (as similar signals do in other systems: Jiggins et al. 2001, 2004; Nokelainen et al. 2011; Twomey et al. 2014, 2016). Females from most *O. pumilio* populations tested spend more time associating with males displaying coloration typical of the female’s population (Summers et al. 1999; Reynolds and Fitzpatrick 2007; Maan and Cummings 2008, 2009; Richards-Zawacki and Cummings 2011). Because there is no evidence of postmating reproductive isolation among Bocas del Toro *O. pumilio* lineages (Summers et al. 2004; Dugas and Richards-Zawacki 2015), these preferences seem the most likely mechanism to prevent gene flow (as is common in young divergences:

Arnqvist et al. 2000; Panhuis et al. 2001; Ritchie 2007; Kraaijeveld et al. 2011). However, remaining untested is the extent to which courtship preferences drive reproductive isolation in sympatry, the key prediction of a speciation-by-sexual-selection argument.

While most distinct *O. pumilio* color morphs occur only in allopatry, there are a few reported cases of sympatry (Dugas et al. 2015), and such populations allow for tests of the hypothesized role of female mate choice in driving and/or maintaining phenotypic diversity (Twomey et al. 2014, 2016). In one polymorphic population, there is some evidence for asymmetric reproductive isolation in the wild, and female preference patterns suggest reinforcement (Richards-Zawacki and Cummings 2011; Richards-Zawacki et al. 2012). However, because the morphs from this polymorphic population do not occur in allopatry today, a full comparison of traits and preferences across a transition zone is not possible (Richards-Zawacki et al. 2012). Here, we address this by comparing female preferences through a phenotypic transition zone in which a polymorphic population occurs between two allopatric, phenotypically distinct, populations of *O. pumilio* (Fig. 1). We began by testing the prediction that females in allopatric populations would prefer males with local coloration. We then asked whether and to what extent morphs are behaviorally isolated in the transition zone by characterizing female preferences of both “pure” phenotypes (individuals phenotypically similar to those from allopatric populations) and the co-occurring phenotypic intermediates. Together, these results will increase our understanding of how phenotypic diversity is maintained and the conditions under which phenotypic divergence does and does not lead to reproductive isolation.

Methods

STUDY SPECIES

Oophaga pumilio is a small (~2 cm snout-vent length), diurnal terrestrial frog that occurs in lowland forests along the Caribbean

side of Central America from Nicaragua to Panama. Males defend territories from which they court females, and females sample males in and around their own, larger, home ranges; both sexes mate multiply (Pröhl and Hödl 1999). Following successful courtship, females lay a clutch of ~5 eggs in the leaf litter, where males may tend the clutch, moistening it daily. Once eggs hatch, one parent, typically but not always the female (Weygoldt 1980; Killius and Dugas 2014), transports tadpoles to water-filled leaf axils. The female then provisions her tadpoles with unfertilized eggs throughout their development (Maple 2002; Dugas et al. 2016).

STUDY ANIMALS

In May–July 2011 and 2012, we collected male and female *O. pumilio* from three mainland populations in the Bocas del Toro region of Panama (Fig. 1). As is the norm for *O. pumilio*, males and females at each site are similar in coloration (Summers et al. 2003). In a monomorphic population near Almirante (09°19'16.3"N, 82°29'49.5"W), frogs are phenotypically similar to ancestral populations (Wang and Shaffer 2008), with red dorsal and ventral coloration and blue legs (Fig. 1). Near Rana Azul, on the north face of the Aguacate peninsula (09°10'37.9"N, 82°16'00.4"W), frogs are monomorphic and entirely blue (Fig. 1). Near Dolphin Bay, on the northern tip of the Aguacate peninsula (9°13'15.70"N, 82°13'5.60"W), both red and blue frogs are present, along with a range of phenotypic intermediates (Fig. 1; Dugas et al. 2015). A mark-recapture survey of 255 frogs at Dolphin Bay indicated that the population contains 6% red, 22% blue, and 72% intermediate frogs (M. B. Dugas, unpubl. data). Although the genetic architecture of coloration in *O. pumilio* remains unknown, captive breeding of several color morphs has demonstrated that coloration in this species is heritable (Summers et al. 2004; Dugas and Richards-Zawacki 2015). The presence of phenotypic intermediates in the red-blue transition zone suggests that coloration is an additive trait in this case; in another polymorphic population, red versus yellow coloration seems most likely to be controlled in large part by dominant/recessive alleles at a single locus (Richards-Zawacki et al. 2012). From Dolphin Bay, we collected frogs that were, by eye, at the extremes of red and blue or most “intermediate”. Differences among these by-eye categories are perceivable in the frog’s visual system and by-eye categorizations are equivalent to more quantitative methods in this (Dugas et al. 2015) and other (Richards-Zawacki et al. 2013) polymorphic *O. pumilio* populations.

We immediately transported field-collected *O. pumilio* to the Smithsonian Tropical Research Institute’s Bocas del Toro field station, where we maintained frogs in plastic enclosures (37 × 22 × 25 cm), separated by sex. Each enclosure housed at most three frogs, each of a different color, to allow identification of individuals. Frogs fed on insects (mostly *Drosophila* spp.) attracted

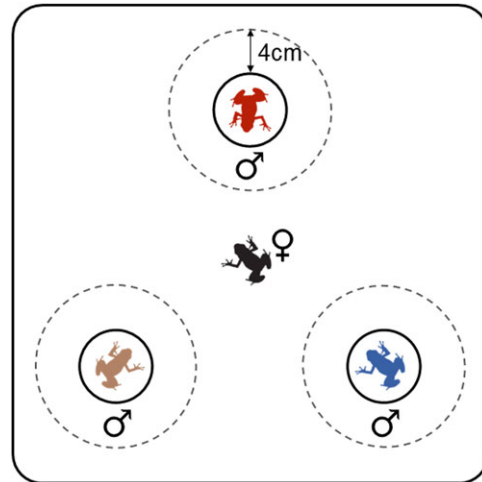


Figure 2. Experimental apparatus used in assays of female preference. During behavioral observations, the three stimulus males were confined under clear plastic domes, and the female was allowed to move freely in the arena.

to fruit placed in their enclosures, and were supplemented with vitamin-dusted termites. Enclosures also contained locally collected vegetation, and were misted daily to maintain humidity. So that we could match males for body size in behavioral assays (see below), we measured mass to the nearest 0.01 g and snout-vent length to the nearest 0.1 mm within one day of capture. We released all individuals following the completion of the experiment.

EXPERIMENTAL DESIGN AND PROTOCOL

Following previous studies in *O. pumilio* (Maan and Cummings 2008; Richards-Zawacki and Cummings 2011), we tested the prediction that females would prefer to associate with males from the same population and/or of the same color using a three-way choice design under laboratory conditions. Each female was simultaneously presented with a male from Almirante (red with blue legs), a male from Rana Azul (entirely blue), and a male with intermediate coloration from Dolphin Bay.

The experimental arena was modified from a similar three-way choice test in Richards-Zawacki and Cummings (2011). The entire arena was a plastic container (60 × 60 × 45 cm) opaque on the sides and covered on top with plastic mesh to allow behavioral observations from above (Fig. 2). The three stimulus males were individually restricted under clear plastic domes ($r = 3.5$ cm $H = 4.5$ cm) placed equidistant from each other (Fig. 2); the position of males from each population was determined haphazardly. During behavioral observations, the focal female was allowed to move freely through the entire arena. All observations were conducted in a dark room, with arenas illuminated by two 60-W halogen bulbs (A19, GE Reveal, USA) and four 75-W UV lights (A19 Blacklight, Koninklijke Philips N.V., Netherlands) covered by

two green-blue gel filters (Lee 728 + CyanGel 4315) to generate lighting conditions similar to that on the forest floor (sensu Maan and Cummings 2008). Males presented simultaneously in trials were matched for snout-vent length (within 1 mm). Males were used in multiple behavioral assays to decrease the total number of animals used in the experiments, but were swapped for newly caught individuals every 7 days to alleviate the possibility of color change in captivity (Summers et al. 2003). Size-matched trios of males were formed upon the day of capture, and remained together throughout the week of experiment. On each experimental day, a male trio was chosen at random, used for assays of four consecutive females, then swapped for a new, randomly selected trio. Unfortunately, records of trio identity were not retained.

We cannot exclude the possibility that male traits other than color influenced the expression of female preference. We can, however, exclude body size, call, tactile, or chemical information, the other traits most likely to shape preference (Dreher and Pröhl 2014), as we size-matched males, no males called during these trials (if they had, we would have excluded these trials from analysis sensu Richards-Zawacki and Cummings 2011), and males were confined under domes.

We placed males in the arena immediately prior to introducing a female. We then placed the female in the arena, equidistant from all three males (Fig. 2), isolated under a dome that was covered with a black visual barrier to prevent her from seeing the males. After 5 minutes, we removed the visual barrier (but not the transparent dome) for 2 minutes before finally lifting the dome from over the female. We observed female behavior for 15 minutes after she first demonstrated interest in a male, operationally defined as approaching within a 4 cm (~2 body lengths) interaction zone while also facing the male (to distinguish interactions from noncourtship movement, sensu Mann and Cummings 2008). If a female failed to demonstrate interest in a male within 15 minutes of her dome being lifted, we terminated the trial and did not include this “nonresponsive” female in further trials or further analyses (sensu Summers et al. 1999; Reynolds and Fitzpatrick 2007; Maan and Cummings 2008, 2009; Richards-Zawacki and Cummings 2011). To avoid any bias introduced by the placement of males, we ran each responsive female through a second trial immediately after the first, rotating the position of males. If a female showed interest in the first but not the second 15 minutes trial, we terminated the experiment and retested the female on a different day.

During the total 30 minutes of observation, we quantified (i) association time, defined as the cumulative time the focal female spent in each of the interaction zones surrounding each male’s dome, and (ii) approaches, defined as the number of times the focal female oriented toward and entered each interaction zone. These two female behaviors we recorded are typically predicted to be positively associated with the probability that a female will

mate with a male in the wild (Summers et al. 1999; Reynolds and Fitzpatrick 2007; Maan and Cummings 2008, 2009; Richards-Zawacki and Cummings 2011); consistent with this assumption, *O. pumilio* courtship in the wild is more likely to result in mating if the female stays in close physical proximity to the male (S. Blomenkamp and H. Pröhl, unpubl. data). We used total association time and approaches during two 15 minute trials for all analyses. Our total sample of responsive females included 30 from Almirante, 29 from Rana Azul, and 90 females from Dolphin Bay (30 each of red, blue, and intermediate phenotypes).

STATISTICAL ANALYSES

To test for female preferences among the three males presented to her simultaneously, we used generalized linear-mixed models (GLMM) with female identity included as a random effect (details below). We tested the main effects of female population-of-origin (Almirante, Rana Azul, Dolphin Bay), stimulus male color (red, blue, intermediate) and the interaction between these two terms. When the interaction term was significant, we separated the three female populations for further analysis, testing only the effect of male color for the two monomorphic populations (Almirante, Rana Azul) and the effects of male color, female color and their interaction in Dolphin Bay models. All analyses were performed in R 3.2.3 (R Development Core Team 2015).

We fitted “approaches” to a GLMM with negative binomial error structure (data were overdispersed when fitted with Poisson) using the *glmmADMB* package (Skaug et al. 2011). We tested the significance of main effects using the “Anova” function in the *car* package, which compares overall model fit with and without a particular effect. We used Tukey’s post hoc tests for pairwise comparisons of approaches to the three stimulus male colors. We fitted “association time” to a linear-mixed model (LMM) using the *lme4* package (Bates et al. 2014). Because of nonnormality of residuals, for hypothesis testing we bootstrapped estimated 95% confidence intervals of the fixed effects and their interaction terms using 5000 iterations. We applied a semiparametric bootstrapping approach using the “bootMer” function in the *boot* package (Canty and Ripley 2012). The LMM was fitted with the Nelder Mean option in “lmer” to improve performance of the semiparametric bootstrap routine. A main effect or interaction was considered significant if the bootstrapped 95% confidence interval did not overlap zero. For post hoc comparisons of the stimulus male colors, we repeated the semiparametric bootstrapping process with a zero intercept model to generate confidence interval for all three levels; in this case, groups were considered significantly different if the 95% confidence intervals did not overlap.

Finally, we asked whether the strength of female approach and association preferences differed among populations. We first determined the preferred male color for females in each of the three populations (see Results). For each individual female, we

Table 1. Generalized linear-mixed model evaluating the effects of stimulus male phenotype, female's population-of-origin and the interaction between the two terms on number of approaches.

Parameters	χ^2	<i>df</i>	<i>P</i> -value
Male phenotype	11.12	2	0.004
Female population-of-origin	15.17	2	< 0.001
Male phenotype × female population-of-origin	31.06	4	< 0.001

Stimulus male population-of-origin: red = Almirante, blue = Rana Azul, intermediate = Dolphin Bay.

Table 2. Linear-mixed model evaluating the effects of stimulus male phenotype, female's population-of-origin, and the interaction between the two terms on association time.

Parameters	2.5% CI	97.5% CI
Male phenotype ¹		
<i>intermediate</i>	−292.23	−72.29
<i>red</i>	−277.27	−48.02
Female population-of-origin ²		
<i>Dolphin Bay</i>	−222.80	−40.12
<i>Almirante</i>	−312.30	−84.74
Male phenotype × female population-of-origin		
<i>intermediate</i> × <i>Dolphin Bay</i>	51.20	298.14
<i>red</i> × <i>Dolphin Bay</i>	197.38	466.99
<i>intermediate</i> × <i>Almirante</i>	109.68	417.42
<i>red</i> × <i>Almirante</i>	292.42	598.66

¹Male phenotype "blue" is the baseline.

²Population "Rana Azul" is the baseline.

Confidence intervals (CI) were 95% percentile bootstrapped. Significance of a term was determined by if the bootstrapped confidence interval overlapped 0. Stimulus male population-of-origin: red = Almirante, blue = Rana Azul, intermediate = Dolphin Bay.

modeled her interest in the male of the population-preferred color, including her "overall preference" (sum of interest in all three males) as an offset term, as some females may be overall more interested in males than others. Approach preference strengths were compared using a generalized linear model (GLM) with quasi-Poisson error structure, including total approaches made by females as an offset term. To avoid normality assumptions, association preference strengths were compared by applying a permutation-based linear model using the "lmp" function of the lmpPerm R package (Wheeler 2010), including total association time as an offset term. Significance was determined using *P*-values calculated from 5000 iterations.

Results

In our initial model that included all observations, we found a significant interaction between female population-of-origin and male phenotype with respect to both association time and approaches (Tables 1 and 2). Because of this significant interaction, we then considered female behaviors separately for each female population-of-origin.

Almirante (red) females spent unequal amounts of time interacting with red, blue, and intermediate males. They spent significantly more time interacting with red males than with blue or intermediate males (bootstrapped 95% CI, red [242.42, 383.01], intermediate [45.84, 109.65], blue [−44.55, 103.12], Fig. 3A). Almirante females also approached the three stimulus males with unequal frequency (GLMM, $\chi^2 = 16.95$, *df* = 2, *P* < 0.001), approaching red males more often than blue males (Tukey post hoc comparisons, red–intermediate: *P* = 0.212, red–blue: *P* < 0.001, intermediate–blue: *P* = 0.171, Fig. 3C).

Rana Azul (blue) females also spent unequal amounts of time interacting with red, blue, and intermediate males. They spent significantly more time interacting with blue males than with red or intermediate males (bootstrapped 95% CI, red [6.99, 137.16], intermediate [−16.22, 125.10], blue [167.83, 300.07], Fig. 3B). Rana Azul females also approached the three stimulus males with unequal frequencies (GLMM, $\chi^2 = 17.15$, *df* = 2, *P* < 0.001). They approached blue males more often than red or intermediate males (Tukey post hoc comparisons, blue–intermediate: *P* = 0.020, blue–red: *P* = 0.015, intermediate–red: *P* = 0.995, Fig. 3D).

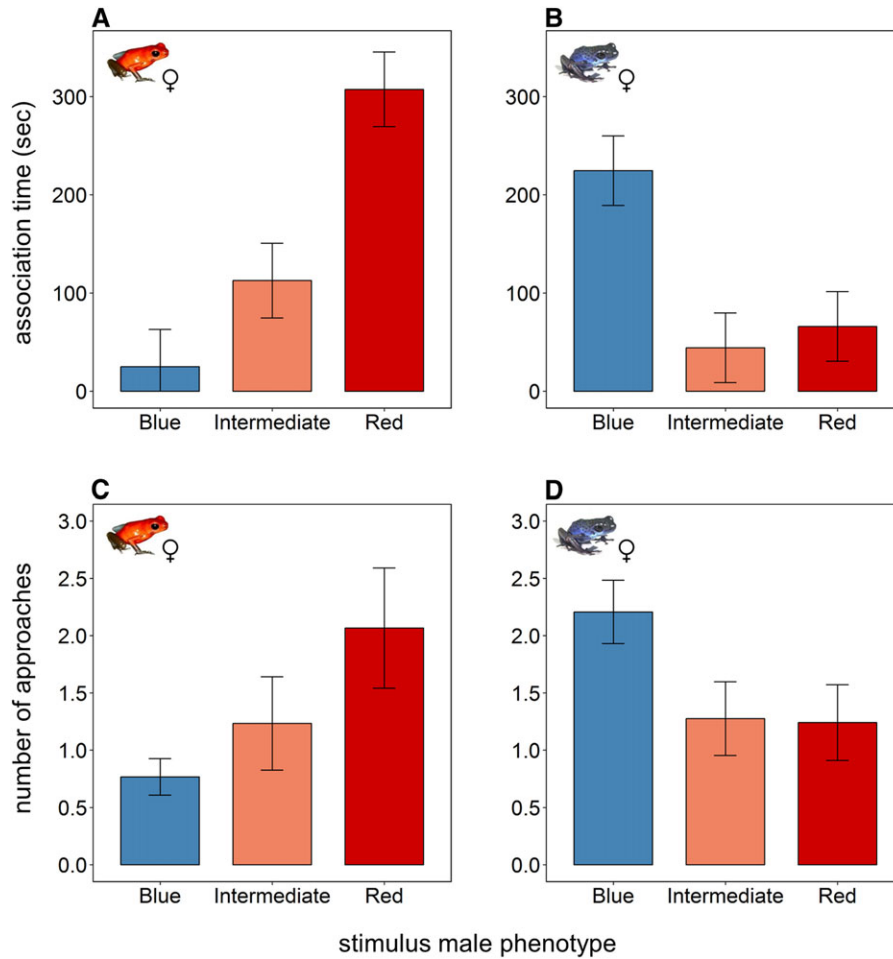


Figure 3. (A, B) Marginal mean (\pm SE) estimated from a linear-mixed model of time female *O. pumilio* from red (Almirante: A) and blue (Rana Azul: B) populations spent with red, blue, and intermediate stimulus males. (C, D) Marginal mean (\pm SE) estimated from a generalized linear-mixed model of approaches female *O. pumilio* from red (Almirante: A) and blue (Rana Azul: B) populations made to red, blue, and intermediate stimulus males.

Table 3. Linear-mixed model evaluating the effects of stimulus male phenotype, female phenotype, and the interaction between the two terms on association time.

Parameters	2.5% CI	97.5% CI
Male phenotype ¹		
intermediate	-101.60	126.62
red	174.97	401.91
Female phenotype ²		
intermediate	-37.28	195.49
red	-103.74	129.09
Male phenotype \times female phenotype		
intermediate \times intermediate	-209.09	128.07
red \times intermediate	-329.80	-2.80
intermediate \times red	-197.44	125.50
red \times red	-357.23	-32.52

¹Male phenotype "blue" is the baseline.

²Female phenotype "blue" is the baseline.

Confidence intervals (CI) were 95% percentile bootstrapped. A term was considered significant if the bootstrapped confidence interval did not overlap 0. Stimulus male population-of-origin: red = Almirante, blue = Rana Azul, intermediate = Dolphin Bay.

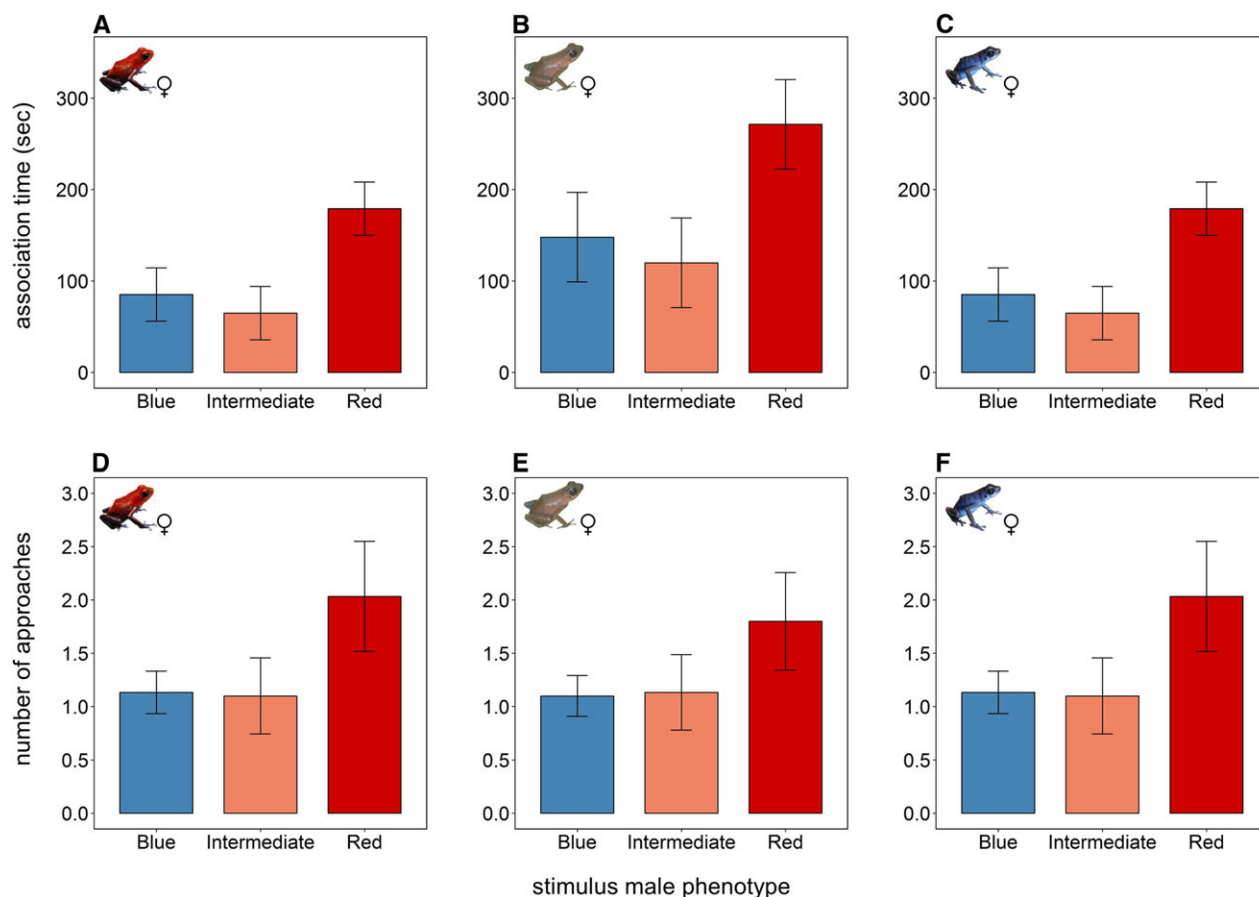


Figure 4. (A–C) Marginal mean (\pm SE) estimated from a linear-mixed model of association time of female *O. pumilio* of red (A), intermediate (B), and blue (C) phenotype from a polymorphic population with blue, red, and intermediate stimulus males. (D–F) Marginal mean (\pm SE) estimated from a generalized linear-mixed model of number of approaches of female *O. pumilio* of red (D), intermediate (E), and blue (F) phenotype from a polymorphic population with blue, red, and intermediate stimulus males.

In Dolphin Bay, the female color \times male color interaction term was significant for comparisons of association time (Table 3), so we tested association preference of each female phenotype separately. Despite a significant interaction term, females of all three colors expressed similar preferences. They spent more time associating with red males than with blue or intermediate males, though only for red and blue females were the 95% CIs nonoverlapping for red versus other male phenotypes (bootstrapped 95% CI, red females: red [128.40, 241.07], intermediate [15.03, 125.68], blue [35.06, 146.73]; intermediate females: red [198.01, 362.13], intermediate [46.49, 208.42], blue [73.84, 237.53]; blue females: red [280.77, 454.86], intermediate [7.17, 182.68], blue [–6.38, 170.09], Fig. 4A–C). The female color \times male color interaction term was nonsignificant for comparisons of approaches (GLMM, $\chi^2 = 0.952$, $df = 4$, $P = 0.917$), and there was no main effect of female color for approaches (GLMM, $\chi^2 = 0.32$, $df = 2$, $P = 0.852$). Dolphin Bay females did, however, approach males of the three colors with different frequencies (GLMM, $\chi^2 = 9.55$, $df = 2$, $P = 0.008$). They approached

red males more often than blue males, and approached intermediate males with frequencies between the two (Tukey post hoc comparisons, red–intermediate: $P = 0.214$, red–blue: $P = 0.005$, intermediate–blue: $P = 0.736$, Fig. 4D–F).

Females from the three populations did not differ in the strength of their preferences for their most preferred male color. The Dolphin Bay females' association preference for red is not significantly different in strength from the Almirante females' association preference for red or the Rana Azul females' association preference for blue (permutation based linear model, $df = 2$, $P = 0.24$). Similarly, the Dolphin Bay females' approach preference for red is not significantly different from the Almirante females' approach preference for red or the Rana Azul females' approach preference for blue (GLM, $LR\chi^2 = 0.837$, $df = 2$, $P = 0.658$).

Discussion

The divergence of mating signals and preference for these signals are necessary for behavioral isolation between differentiated

lineages (Panhuis et al. 2001; Ritchie 2007). We found evidence suggesting that coloration and female preferences have indeed diverged in concert in the monomorphic *O. pumilio* populations: females from the pure blue and red populations spent more time with males from their own population and approached them more often. In the transition zone, however, instead of favoring males similar to their own color, red, blue, and intermediate females all preferred red males, and this preference was similar in strength to the preferences observed monomorphic populations. While divergent preferences may or may not have contributed to the initial divergence in coloration between morphs, they would be critical to limiting gene flow in sympatry. At least for these two color morphs, assortative preferences are expressed in allopatry but not in sympatry, and are thus unlikely to bring about reproductive isolation. Similar breakdowns of preferences expressed in allopatry have been documented in grasshoppers (*Chorthippus parallelus parallelus*, *C. p. erythropus*: Ritchie et al. 1989), wood rats (*Neotoma bryanti*, *N. lepida*: Shurtliff et al. 2013) and fruit flies (*Drosophila subquinaria*, *D. recens*: Bewick and Dyer 2014), indicating that divergent traits and preferences often do not contribute to reproductive isolation in sympatry.

Although allopatric divergence in coloration is the norm in *O. pumilio* (Gehara et al. 2013), distinguishing between primary divergence and secondary contact in a polymorphic zone is notoriously difficult (Barton and Hewitt 1985). It is possible that the red-blue polymorphic population we studied resulted from (i) secondary contact between red and blue lineages that diverged in allopatry, (ii) the blue phenotype first arising in a monomorphic red population in the Northern tip and spreading to fixation on the rest of Aguacate peninsula, or (iii) the red phenotype arising from within the monomorphic blue population in Northern Aguacate, independent of the Almirante red phenotype (i.e., a reversion to a phenotype similar to the ancestral one). Given the likely alternating periods of connectivity and vicariance in this archipelago, along with current distribution of distinct *O. pumilio* color morphs, secondary contact seems the most parsimonious explanation for the red-blue transition zone. Regardless of how the transition zone arose, the global preference for red in the polymorphic population suggests that color and color preference have evolved at different speeds in red and blue lineages. We cannot exclude the possibility that male traits other than color cause transition zone females to prefer red stimulus males (although our methods allow us to exclude body size, call, and tactile or chemical cues). If this is the case, although unlikely, our results would similarly suggest that the unknown trait and corresponding preference evolved at different speeds.

Assuming secondary contact, it remains unclear why blue females in the polymorphic population expressed a preference different from blue females in the allopatric population, and why female preference in the polymorphic population converged on

red. Myriad mechanisms can initiate the codivergence of traits and preferences in isolated populations (e.g., sensory drive, ecological adaptation, genetic drift), but the association between preference and trait loci can easily be broken down by recombination (Otto et al. 2008; Servedio and Bürger 2014). Recombination is likely in *O. pumilio* given that female preferences are clearly not absolute (Summers et al. 1999; Reynolds and Fitzpatrick 2007; Maan and Cummings 2008; Dugas and Richards-Zawacki 2015). The preference for red displayed by females from the polymorphic population may be advantageous if females accrue direct or indirect benefits from mating with a red male in Dolphin Bay, for example if red males are better brood tenders, or sire healthier tadpoles (a possibility suggested by among-morph differences in male reproductive success and tadpole performance: Dugas and Richards-Zawacki 2015; Dugas and Richards-Zawacki 2016).

A universal preference for red in the transition zone could also emerge without any changes in the genetic mechanism underlying mate preferences. Within populations, *O. pumilio* females prefer brighter males over duller ones (Maan and Cummings 2009), a pattern that might explain a universal preference for the brighter red males over the duller blue and intermediate ones in the transition zone (Rudh et al. 2011; Maan and Cummings 2012; Dreher and Pröhl 2014). However, this explanation is inconsistent with the finding that females from several *O. pumilio* lineages (including the Rana Azul females tested here) prefer males from their own population even when offered males from brighter allopatric populations (Summers et al. 1999; Reynolds and Fitzpatrick 2007; Maan and Cummings 2008). It remains possible that color-based female preferences are shaped by the integration of independent assessments of brightness and color (i.e., hue) or that color is simply important along a gradient of familiar to unfamiliar. Mate preferences might also be plastic, with convergent female preferences in the polymorphic population reflecting shared natural and/or social environments (Svensson et al. 2010; Kozak et al. 2011); rather than selection on preferences driving the preference for red, this scenario allows females to learn that some phenotypes make better mates (Rodríguez et al. 2013). Sexual imprinting based on social interactions can create positive frequency-dependent selection that results in females shifting to prefer the most common male type (Rodríguez et al. 2013). However, red is the rarest phenotype in Dolphin Bay (~6%), suggesting alternative mechanisms. Females may also simply learn to prefer the rarest male phenotype; while this “rare-male effect” has been documented in several taxa, the proximate mechanism is poorly understood (Singh and Sisodia 1999; Eakley and Houde 2004). The effect of behavioral learning on population divergence is dependent upon the cue for learning, and the tutor from whom females learn (Verzijden et al. 2012; Yeh and Servedio 2015). Asymmetric responses to diverged sexual signals are commonly observed between young lineages (Hardwick et al. 2013, Martin

and Mendelson 2013; Shurtliff et al. 2013). Identifying the mechanisms shaping female preferences will be key to predicting the speed and direction of evolution when divergent lineages interact and understanding the full complement of selective pressures shaping and maintaining phenotypic diversity (Verzijden et al. 2005).

The high frequency of intermediate individuals in the red-blue transition zone suggests frequent among-morph matings in the wild, and unless some cost prevents red males from mating with the phenotypically diverse females that prefer them, such gene flow should continue. There is no evidence that between-lineage matings are any less productive than within-lineage matings in *O. pumilio* (Dugas and Richards-Zawacki 2015) or that natural selection penalizes phenotypic intermediates (Richards-Zawacki et al. 2013; Yeager et al., unpubl. ms.). If female preferences for red males drive mate choice, the frequency of both red and intermediate phenotypes should increase over time, a possibility consistent with our repeated sampling at Dolphin Bay (Y. Yang and M. B. Dugas, unpubl. data) and one that can be tested with continued monitoring of the entire transition zone. Equilibrium in morph frequency could be maintained by natural selection against red, for example if local predators must learn to associate the frogs' toxicity with color, red may be a less effective aposematic signal than the more common blue or intermediate morphs (Ruxton et al. 2004). All current evidence suggests that no such natural selection costs occur in *O. pumilio* populations (Hegna et al. 2013; Richards-Zawacki et al. 2013; Dreher et al. 2015; Yeager et al. unpubl. ms.). However, it is possible that the results of all these studies are influenced by recent anthropogenic disturbance that has altered the selection regimes on aposematic coloration, resulting in collapse of trait and preference differentiation perhaps driven and/or maintained by natural selection.

The collapse of divergent within-species lineages is much more common than speciation (Rosenblum et al. 2012; Dynesius and Jansson 2013), and asymmetry in the strength of assortative mating among lineages (Hardwick et al. 2013; Martin and Mendelson 2013; Shurtliff et al. 2013) has been suggested as a common reason for collapse instead of progression to full reproductive isolation (Arnold et al. 1996; Servedio and Bürger 2014). While divergent traits and preferences are necessary to drive reproductive isolation, they are not necessarily sufficient to do so or to move lineages towards speciation (Jennions and Petrie 1997). Studies of Bocas del Toro *O. pumilio* lineages can continue to contribute to our understanding of the role of female preferences in driving behavioral isolation, in particular if focus is directed toward: (i) identifying the proximate mechanisms by which female mate preferences are shaped, including the relative contribution of genetics and plasticity, (ii) identifying the factors that shape and constrain the relationship between preferences expressed in the lab and actual mate choice in the wild, including

the roles not only of female choice but also of male–male competition (Qvarnström et al. 2012), and (iii) continued monitoring of phenotype frequency in contact zones, the natural laboratory that allows the rare but critical test for reproductive isolation in nature. Our findings highlight the complexity in the evolution of behavioral isolation, and the need for future studies to investigate the circumstances under which divergent preferences do and do not contribute to speciation.

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DATA ARCHIVING

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's website:

Figure S1. Individual variation in relative interest that *Oophaga pumilio* females showed in a stimulus male of the color globally preferred by females in her population.