



Variation in Melanism and Female Preference in Proximate but Ecologically Distinct Environments

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Introduction

The study of vertebrate pigmentation is a growing area of evolutionary biology research, because it at once unites the fields of behavior, ecology, physiology, molecular genetics, and development in a framework for understanding evolutionary processes across levels of biological organization (Hoekstra 2006; Price et al. 2008; Kronforst et al. 2012). While conspicuous phenotypes may come at a physiological cost (Price et al. 2008) or increase detection by visual predators (Majerus et al. 2000), they can also increase fitness by

Abstract

Variation in pigmentation traits is an effective window to evolutionary processes due to their importance for survival and reproduction. In particular, one of the leading hypotheses for the maintenance of conspicuous pigmentation in natural populations is its signaling function in mate choice. Here, we demonstrate the occurrence of melanism in poeciliid fishes of the genus *Poecilia* that inhabits toxic, hydrogen sulfide springs in southern Mexico and the absence of melanism from closely related populations in reference habitats lacking hydrogen sulfide. Assays of female mate preference in both habitat types were used to examine whether divergence in female preference for melanism contributes to its maintenance in hydrogen sulfide springs. We found significant variation in female preferences for melanistic males. Specifically, melanistic females from the toxic spring exhibited a significant preference for melanistic males, while non-melanistic females from the same population exhibited no preference. Females from the non-sulfidic reference population discriminated strongly against melanistic males. Preferences of melanistic females appear to be a significant force in the maintenance of melanism in sulfidic habitats and suggest a change in preference as the divergence from non-sulfidic ancestors. Potential polymorphism in preference within the hydrogen sulfide spring indicates that preference for melanistic males may not be environmentally controlled. Thus, a change in preference following divergence can promote the maintenance of variation in pigmentation within populations and between divergent habitats.

providing a reproductive advantage, if mating partners exhibit a preference for the conspicuous trait (Hughes et al. 1999). A diverse range of taxa exhibit female preferences that contribute to the maintenance of polymorphisms in pigmentation. Examples include plumage characteristics in birds (Hill 1990; Jawor et al. 2003), color polymorphic lizards (Perez i de Lanuza et al. 2013), and coloration in fish (Seehausen & van Alphen 1998; Kingston et al. 2003; Takahashi & Hori 2008; Hughes et al. 2013). The maintenance of pigment polymorphisms often depends on a balance between a variety of abiotic

environmental, ecological (i.e., biotic interactions), and sexual sources of selection. For example, pigment patterns in guppies are under selection by female choice and predators (Endler 1983), while melanism in the rock pocket mouse, *Chaetodipus intermedius*, appears to have evolved primarily as a response to ecological selection favoring individuals that match their environment (i.e., crypsis) presumably to reduce detection by predators with less or no influence of female mate choice (Hoekstra et al. 2005).

Color polymorphisms are common in livebearing fishes in the family Poeciliidae. Probably the best-documented case pertains to female choice for conspicuous color and pattern variation in the guppy, *Poecilia reticulata* (Endler 1980; Houde 1987). Female guppies prefer males with conspicuous phenotypes (Hughes et al. 1999; Eakley & Houde 2004), but visual predators provide a significant source of selection against male color patterns that do not match the environmental background (Endler 1980, 1983). Thus, guppy color patterns have evolved as a result of a balance between natural and sexual selection. A variety of other poeciliids, including species of the genera *Gambusia*, *Xiphophorus*, and *Poecilia*, also exhibit variation in the presence of melanistic spots, which can cover the body in varying degrees (Regan 1961; Borowsky 1978; Angus 1983; Coleman et al. 2009). Studies in these species have documented both natural and sexual selection for and against such melanism (Horth 2003, 2004; Culumber & Rosenthal 2014). Hence, conspicuous pigmentation can be maintained despite potential predatory or physiological costs, if it positively affects the reproductive success of carriers.

Color patterns often fall within one of two classes: those that are polymorphic and those that are variable. Polymorphic traits typically refer to coloration that can be characterized as discrete phenotypes. Other color traits exhibit continuous variation in degree of coloration around a mean value rather than discrete phenotypes. These two classifications are not mutually exclusive, because coloration can be both polymorphic and show variation in degree of coloration (Roulin 2004). Color patterns of poeciliid fishes provide a great example of these types of color variation. For example, coloration in guppies represents both polymorphism and continuous variation. The widely studied orange spots found on male guppies are a polymorphic color pattern with a heritable basis (Houde 1992), but this trait as well as melanistic spots present in guppy males also exhibit continuous variation in spot size (Endler 1983). Of particular relevance to this study is polymorphism in melanism, given that these traits are commonly controlled by a single gene

or gene complex and often also associated with behavioral and/or physiological traits (Ducrest et al. 2008). Here, we studied the occurrence of melanistic spotting and the forces maintaining polymorphism in this trait within and between a series of proximate populations of poeciliid fishes in the genus *Poecilia*. In the present manuscript, we focus on polymorphism in spotting, that is, the presence or absence of melanistic spots, given the typically large effects of a few genes and integration of phenotypes in vertebrates (Ducrest et al. 2008).

Livebearing fishes of the genus *Poecilia* have evolved in divergent habitats along a physicochemical gradient. *Poecilia mexicana* is a widespread species in streams of southern Mexico, and evolutionary independent lineages have colonized and adapted to toxic springs rich in hydrogen sulfide (H_2S) in different river drainages (Tobler et al. 2011; Palacios et al. 2013). Populations in sulfidic habitats are both genetically and phenotypically differentiated from populations in adjacent non-sulfidic habitats (Tobler et al. 2011). While recent work showed that reproductive isolation between populations in different habitat types is at least in part mediated by natural and sexual selection against immigrants, the traits that play a part in visual discrimination against migrants remain unclear (Plath et al. 2013). Multiple studies in this system have documented variation in female preferences among populations and habitat types for morphological traits (Tobler et al. 2009a,b; Plath et al. 2010, 2013; Bierbach et al. 2013). However, the potential for variation in female preferences both among and within populations has not yet been considered. Here, we report the occurrence of polymorphism in a conspicuous visual trait, melanistic coloration (Fig. 1), in *Poecilia* across a physicochemical environmental gradient. Using frequency data from sulfidic and non-sulfidic habitats as well as tests of association preferences among female types, we investigate whether sexual selection plays a role in the presence and absence of melanism in sulfidic and non-sulfidic habitats, respectively.

Methods

Study System and Specimen Collection

Melanism was studied in sulfidic and non-sulfidic populations of *Poecilia mexicana* in southern Mexico. The species is common in non-sulfidic streams, but has also colonized H_2S -rich springs in four different tributaries of the Rio Grijalva (Tobler et al. 2011; Palacios et al. 2013). Geographic and molecular

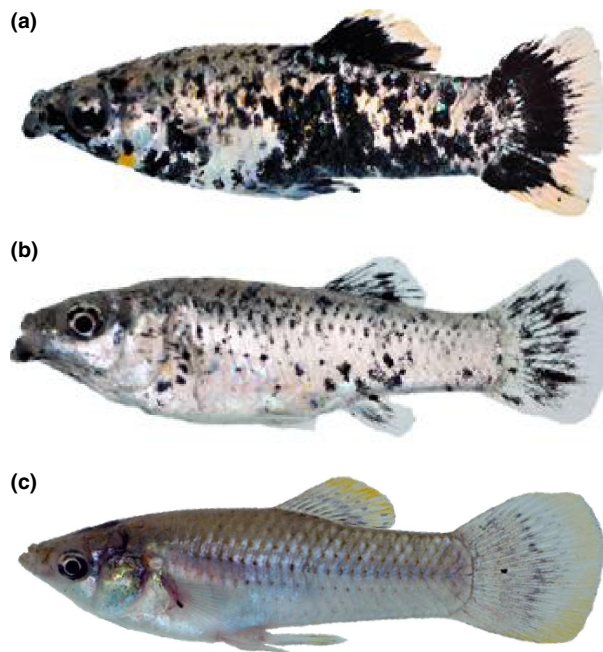


Fig. 1: Three fish from a sulfidic habitat represent the range of melanism observed within populations. In this study, any fish with melanistic spots was considered melanistic and those with no spots were non-melanistic: (a) melanistic male with dense spotting, (b) melanistic female with sparse spotting, and (c) non-melanistic male with no spots. Note: Images are not to scale, but rather are to provide a reference for the visual difference in melanism among types.

genetic evidence indicates at least three (possibly four) evolutionary independent colonizations of sulfide springs in this region (Palacios et al. 2013). The sulfide springs in the two western-most drainages (Pichucalco and Ixtapangajoya) harbor the oldest (approx. 300 000 years; Pfenninger et al. 2014) and most distinct lineages of sulfur spring fish. These sulfur spring populations have been described as highly endemic, distinct species: *P. sulphuraria* in the Pichucalco and *P. thermalis* in the Ixtapangajoya drainage (Palacios et al. 2013 for a review). In contrast, the sulfide springs in the Puyacatengo and Tacotalpa drainages have been colonized more recently (<50 000 years ago; Pfenninger et al. 2014). It is important to emphasize, however, that differences in taxonomic status are not reflective of biological realities in these systems. All sulfide spring populations investigated to date are morphologically distinct (whether they are described as separate species or not), show some degree of reproductive isolation from proximate populations in non-sulfidic habitats, and even populations in sulfide spring described as distinct species are nested within the phylogeny of the widespread *P. mexicana* (Plath et al. 2007; Tobler et al. 2011; Palacios et al. 2013; Plath et al. 2013).

For calculation of the frequency of melanism in different populations, we used field notes on the number of melanistic and non-melanistic individuals collected by seine between 2006 and 2013 from sulfidic and non-sulfidic sites in three drainages (Tacotalpa, Puyacatengo, and Pichucalco; Table 1). Any fish that had a melanistic spot was classified as melanistic, while fish that had no spots were classified as non-melanistic (Fig. 1). All collections were made with a 3×1.2 m seine with 4-mm mesh size. The total time spent seining (sampling effort) would affect the total abundance of fish sampled at different localities and within localities across years. However, variation in sampling effort should not affect the discovery rate (proportions) of melanistic and non-melanistic fish within each sample. Sampling effort should therefore not influence the observed frequency of melanistic fish among sites nor within sites across years.

To test for mating preferences for or against melanistic males, we focused on a sulfidic and a non-sulfidic population in the Pichucalco drainage, which are separated by <4 river kilometers. Female and male sulfidic ecotypes (*P. sulphuraria*) were collected by seine from La Gloria, a hydrogen sulfide-rich spring complex (Tobler et al. 2011). Females and males from the non-sulfidic ecotype (*P. mexicana*) were collected by seine from a non-sulfidic, freshwater reference site (Río El Azufre, west branch; Tobler et al. 2011), which connects downstream from the sulfide springs. Detailed information on these collection localities can be found in Tobler et al. (2011). After collection, males and females were housed in population-specific 40-l containers and allowed to acclimate to laboratory conditions for at least 48 h prior to testing. All fish were fed in the morning prior to testing.

Experimental Set-up and Association Trials

The testing tanks for association trials consisted of a central 20-l aquarium ($40 \times 20 \times 25$ cm) constructed from UV transparent plexiglass filled halfway with clean tap water that had been bubbled vigorously to allow any residual chlorine to gas out of the water prior to use. The central tank was divided into three zones—a central neutral zone 20 cm wide and two 10 cm ‘response zones’ at either end of the tank—by marking the outside of the tank in a manner visible only to the human observers to facilitate the scoring of association time. Association time was scored as the time that a female was within the 10-cm response zone closest to a given male. Live stimulus males differing in the presence (melanistic) and absence (non-melanistic) of black spots were presented in

Table 1: The frequency of melanistic fish in collections made over multiple years in sulfidic and non-sulfidic reference habitats across three river drainages. Means represent the frequency of melanistic fish in all sulfidic and non-sulfidic sites, regardless of whether melanistic fish have been observed, and within each habitat type only for those populations in which melanistic fish have been observed (sulfidic and non-sulfidic 'with melanism')

Locality	Drainage	Type	Aug. 2004		Jan. 2006		April 2007		Jan. 2008		Aug. 2008		Feb. 2009		July 2010		Sept. 2010		July 2011		Mar. 2012		June 2012		
Bonita	Tacotalpa	Non-sulfidic	-	0/56	0/44	0/68	0/51	0/23	0/29	0/23	1/28 (0.0357)	0/29	-	-	0/36	0/38									
Tacubaya	Tacotalpa	Non-sulfidic	-	-	0/33	0/20	0/36	1/28 (0.0357)	-	-	-	-	-	0/5	0/28										
Tres	Tacotalpa	Non-sulfidic	-	0/17	0/26	0/29	0/11	0/19	0/21	0/19	0/21	-	-	-	-										
EAI	Tacotalpa	Sulfidic	0/100	0/44	0/117	0/38	0/36	0/52	0/61	0/52	0/61	0/15	0/41	0/29											
EAI	Tacotalpa	Sulfidic	-	0/15	0/42	0/72	0/43	0/30	-	0/30	-	-	-	-	0/21										
La Lluvia upstream	Puyacatengo	Non-sulfidic	-	-	0/41	-	0/68	0/19	-	0/19	-	0/41	-	-	-										
Puyacatengo road crossing	Puyacatengo	Non-sulfidic	-	-	0/36	-	0/13	0/22	-	0/22	-	-	-	-	-										
Puyacatengo at Vincente	Puyacatengo	Non-sulfidic	-	-	0/72	-	0/37	-	0/37	-	-	-	0/24	0/33											
Guerrero	Puyacatengo	Sulfidic	-	-	7/51 (0.137)	-	8/64 (0.125)	3/37 (0.0811)	1/94 (0.0106)	1/94	-	-	-	-	6/46 (0.130)										
La Lluvia small spring	Puyacatengo	Sulfidic	-	-	0/34	-	-	0/9	0/18	0/18	-	-	-	-											
La Lluvia big spring	Puyacatengo	Sulfidic	-	-	0/23	-	0/21	0/13	0/7	0/7	-	-	0/5	-											
Rafael	Pichucalco	Non-sulfidic	-	-	0/17	-	1/52 (0.0192)	0/22	0/42	0/42	1/39 (0.0256)	-	1/49 (0.0204)	0/35											
Rosita	Pichucalco	Non-sulfidic	-	-	0/31	-	0/68	0/47	0/31	0/31	0/18	0/43	0/27	0/67											
Vet Station	Pichucalco	Non-sulfidic	-	-	0/129	-	1/108 (0.009)	0/87	0/78	0/78	2/28 (0.0714)	0/40	0/42	1/94 (0.106)											
Banos	Pichucalco	Sulfidic	1/67 (0.0149)	-	4/58 (0.0689)	-	11/98 (0.112)	-	7/52 (0.135)	3/35 (0.0857)	4/29 (0.138)	4/60 (0.0667)	9/53 (0.170)												
La Gloria	Pichucalco	Sulfidic	-	-	4/58 (0.0689)	-	11/98 (0.112)	-	7/52 (0.135)	3/35 (0.0857)	4/29 (0.138)	4/60 (0.0667)	9/53 (0.170)												
Means	Global Sulfidic																								
	Global Non-sulfidic																								
	72/2167 (0.0332)																								
	4/1718 (0.00232)																								
	Sulfidic with melanism																								
	72/1350 (0.0533)																								
	Non-sulfidic with melanism																								
	4/406 (0.00985)																								

separate aquaria of equal width and height placed flush against either side of the central testing tank. This allowed females to assess visual but not chemical cues. The walls of the room used for testing were white to maximize contrast of the background with the males in the stimulus tanks. The room was illuminated with ambient light from windows in part of the room and supplemented with standard overhead fluorescent lights. As melanistic individuals are exceedingly rare in non-sulfidic habitats, all stimulus males were of the sulfidic ecotype to eliminate any effects of phenotypic differences between ecotypes on female preferences. While sulfidic females were more familiar with the stimulus males than non-sulfidic females, we compared female preferences for the two male phenotypes only within ecotypes, thus avoiding any potential noise stemming from differences in motivation to choose between males due to familiarity. Furthermore, non-sulfidic females discriminated between males differing in melanism, suggesting that lack of familiarity with sulfidic ecotype males as a whole is unlikely to have affected the results.

Preferences for melanistic and non-melanistic males were tested using association time as a proxy. Association time is a standard measure for mate choice in poeciliid fishes (McLennan & Ryan 1997; Bisazza et al. 2001; Kahn et al. 2009; Verzijden et al. 2012; Plath et al. 2013). In poeciliids, association times are repeatable preference metrics (Cummings & Mollaghan 2006) and are predictive of actual reproductive outcomes (Walling et al. 2010).

Three female types were used in association trials: non-melanistic sulfidic ecotypes ($N = 17$), melanistic sulfidic ecotypes ($N = 18$), and non-melanistic non-sulfidic ecotypes ($N = 11$). Only non-melanistic females were tested from the non-sulfidic reference site, because melanistic fish were very rare in that habitat type (Table 1). For each trial, a focal female was introduced into the central tank and one male into each of the stimulus tanks. Fish were allowed to acclimate for 10 min. Opaque dividers were placed between the stimulus tanks and the central tank to prevent visual contact during the acclimation period. After 10 min, the dividers were gently removed and the trial began. Female association time with each stimulus was recorded for 5 min using handheld timers. The tanks containing the stimulus males were then swapped to control for side biases, and association time was recorded for another 5 min. As female poeciliids often exhibit preferences for males with larger body size, melanistic and non-melanistic male stimuli were size-matched for each trial to within approx. 2 mm as in a previous study of this system

(Plath et al. 2005), but were otherwise chosen haphazardly for each trial. As females may be more motivated to respond to active stimulus males compared with males that do not move, we initially recorded male activity (active or not active) every 30 s of each trial. However, activity rates of non-melanistic and melanistic males across the first 42 five-min trials ($N = 420$ observations for each male type) were 98.8% and 97.6%, respectively. We can therefore rule out differences in male activity as a driver of female association.

Statistical Analyses

Data were first checked and met the assumptions of normality and homogeneity of variance prior to conducting analyses. To statistically test whether the frequency of melanism differs between habitat types, we pooled samples from different sites and visits into categories reflecting habitat type (sulfidic or non-sulfidic) and drainage (Tacotalpa, Puyacatengo, and Pichucalco). For each drainage, frequencies of melanistic fish were then compared between sulfidic and non-sulfidic habitats using Fisher's exact test.

Differences in female association time with male stimuli were assessed in two complementary ways. First, we used a repeated measures analysis of variance (rmANOVA) to assess whether preferences for melanistic males differed among female types (sulfidic melanistic, sulfidic non-melanistic, and non-sulfidic). Female type was the independent variable, and the total time females spent with the melanistic male and total time spent with the non-melanistic male in the two side-swapped trials were the repeated measures. We then independently conducted individual paired *t*-tests on total time spent with the melanistic male compared with total time with the non-melanistic across the two trials within each of the three female types. These tests were used post hoc to assess whether specific female types had preferences for or against melanistic males, as the repeated measures ANOVA can only detect difference in association time among female groups not differences in preference between male stimuli within female groups. All tests were two-tailed.

Results

Frequency of Melanism

Melanistic individuals are consistently recorded at low frequency in some sulfidic habitats, but are only rarely observed in, and are typically absent, from

non-sulfidic habitats (Table 1). Interestingly, not all sulfidic habitats have melanistic fish. Melanism was not observed in either of the sulfidic habitats in the Tacotalpa drainage despite annual collections dating back to 2004, which is reflected in the lack of significant differences in the frequency of melanism between habitat types of that drainage (Fisher's exact test: $p = 0.45$; see Table 2 for contingency tables). In contrast, melanism was significantly more common in sulfidic habitats in the Puyacatengo ($p < 0.001$) and the Pichucalco ($p < 0.001$) drainages, with overall melanism frequencies ranging between 4.6% and 5.7% in sulfidic springs. In contrast, melanistic individuals in non-sulfidic habitats of all drainages occurred at a frequency $< 0.5\%$.

Female Association Preferences

Seventy-two percent of melanistic females and 58% of non-melanistic females from the sulfidic habitat showed a net preference for the melanistic compared to the non-melanistic male. In contrast, all females from the non-sulfidic habitat exhibited a net negative preference for (i.e., discrimination against) the melanistic male. Accordingly, the analysis revealed a significant interaction between the repeated measure (association time with either stimulus) and female type (Table 3). Post hoc tests revealed that preferences of females from the non-sulfidic population differed from both sulfidic female types ($p < 0.01$), but female

Table 2: Contingency tables from Fisher's exact test. The observed and predicted (in parentheses) frequencies of non-melanistic and melanistic individuals are given for each drainage and habitat type

Drainage	Habitat type	Non-melanistic	Melanistic
Tacotalpa	Non-sulfidic	617.0 (617.6)	1.0 (0.4)
	Sulfidic	756.0 (755.4)	0.0 (0.6)
Puyacatengo	Non-sulfidic	443.0 (432.4)	0.0 (10.6)
	Sulfidic	334.0 (344.6)	19.0 (8.4)
La Gloria	Non-sulfidic	654.0 (637.8)	3.0 (19.2)
	Sulfidic	1011.0 (1027.2)	47.0 (30.8)

Table 3: Results from repeated measures ANOVA on net association with a melanistic male compared with a non-melanistic male. Female *Poecilia mexicana* from the non-sulfidic ecotype had significantly different preferences from the two female types from the sulfidic spring. Significant values are indicated in bold

Source	df	$\bar{\chi}^2$	F	p-value
Repeated measure (trial)	1	6306.098	0.631	0.431
Repeated measure*Female type	2	60361.513	6.037	0.005
Error	43	9999.371		

types within the sulfide spring did not differ significantly in their preference ($p > 0.05$). Females from the sulfidic habitat exhibited a positive mean net preference ($+39 \pm 24$ s; \pm SE) for the melanistic male stimuli, while females from the non-sulfidic habitat exhibited a strong bias against the melanistic males (-229 ± 38 s).

Individual t-tests provided additional insights into the nature of female preferences for melanistic males in each female type. Melanistic females from the sulfide spring significantly preferred melanistic males over non-melanistic males ($t_{17} = 3.01$, $p = 0.008$; Fig. 2). Non-melanistic females from the sulfide spring exhibited no preference for either male type ($t_{16} = 0.99$, $p = 0.33$; Fig. 2). In contrast, females from the nearby non-sulfidic reference population exhibited a significant and strong preference against melanistic males ($t_{10} = 6.02$, $p < 0.001$; Fig. 2), associating with non-melanistic males an average of four times longer (70 ± 14 s compared to 299 ± 27 s).

Discussion

Melanistic individuals are extremely rare in non-sulfidic populations but persist in several sulfide spring populations of *Poecilia mexicana*, particularly in the Puyacatengo and the Pichucalco drainages. Overall,

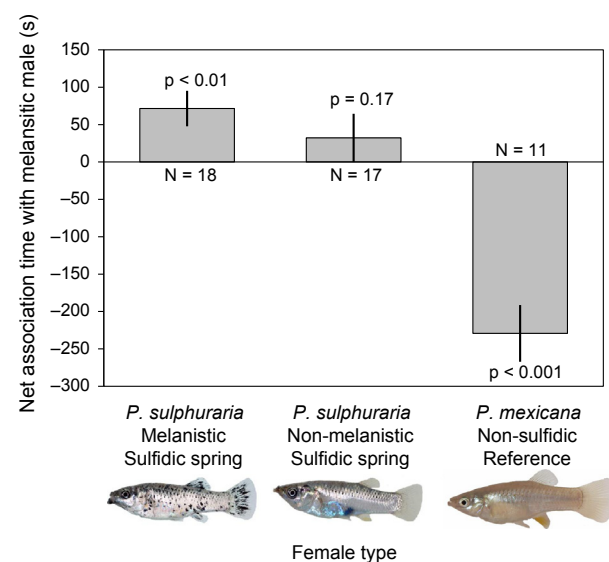


Fig. 2: Net association time (time with melanistic minus time with non-melanistic; \pm SE) of three female types with melanistic and non-melanistic males. Melanistic females from the sulfide spring preferred melanistic males ($p < 0.01$) as revealed by paired t-tests on repeated trials for each female, while *Poecilia mexicana* from the adjacent reference locality discriminated strongly against them ($p < 0.001$). Images are not to scale.

the frequency of melanism was relatively low, even within sulfidic sites (<5%). The frequency melanism in this system appears to be intermediate among other poeciliids: only 1% of *G. holbrooki* exhibit a melanistic phenotype (Horth 2003), but up to 40% of individuals within some populations *X. variatus* exhibit some form of melanism (Z. W. Culumber, unpubl. data). Our findings suggest that the maintenance of melanism in sulfidic habitats is associated with a change in female preference as colonization of sulfidic habitats, which is supported by the difference in preference between females of the two habitat types. Given the strong discrimination against melanistic males by females from the non-sulfidic reference site, melanism could contribute to reinforcement and reproductive isolation between adjacent sulfidic and non-sulfidic populations if the trait increases in frequency, because melanistic males likely would have highly reduced reproductive success in non-sulfidic sites. Behavioral processes, and specifically a change in mating preference during the evolution of these populations, provide a robust mechanism for the persistence and absence of melanism in divergent habitat types and facilitate the stable maintenance of pigmentation polymorphism in sulfidic populations. Although previous studies have reported variation in female preferences among habitat types and populations, this is the first study to demonstrate variation in female preferences within a single population.

The origin and evolution of conspicuous traits and preferences for them remains a central question in evolutionary biology and behavioral ecology (Roulin 2004; Gray & McKinnon 2007). Our results suggest that there is a polymorphism in female preference that originated within the sulfidic population following divergence from the non-sulfidic *P. mexicana* ancestor. As in *Gambusia holbrooki* (Bisazza & Pilastro 2000), we observed variation in female preference for melanism among populations. However, their variation in female preferences was documented between a native and introduced population on separate continents. Here, the change in female preference has occurred across a steep physicochemical barrier and over a short geographic distance. We cannot entirely rule out the possibility that the preference originally arose in melanistic females in the non-sulfidic population prior to colonization of sulfur habitats, but the extreme rarity of melanistic fish in non-sulfidic habitats prevented testing this hypothesis in the present study.

It remains to be studied in detail how genetic variation and environmentally induced phenotypic plasticity shape variation in mating preferences and in the

occurrence of melanism. The variation in preference among females within the sulfidic habitat—depending upon whether females themselves are melanistic—indicates that the preference likely does not have a strictly environmental basis. Similarly, the lack of melanism in the Tacotalpa drainage suggests that melanism is not solely under environmental control (i.e., through effects of hydrogen sulfide), otherwise it would be expected that melanistic phenotypes consistently occur in sulfidic environments. Finally, there is circumstantial evidence that the occurrence of melanism has at least a partially heritable basis. Melanistic individuals also occur in common garden raised laboratory stocks. This is particularly evident randomly outbred stocks of the La Lluvia population from the Puyacatengo drainage, and a single melanistic female collected from a non-sulfidic site in the Pichucalco drainage also gave birth to predominantly melanistic fry in the laboratory (M. Tobler, pers. obs.).

Although association preferences documented in our study theoretically could be a consequence of either mating or shoaling preferences in the different female groups investigated (Gabor 1999), shoaling preferences are unlikely drivers of the documented patterns both because of evidence from previous research and inconsistencies with *a priori* predictions. Our methods closely followed those of published mate choice experiments in this system (Tobler et al. 2009a,b; Plath et al. 2013), and association time is a standard metric of mate choice in poeciliids predicting reproductive outcomes (Walling et al. 2010) and mate choice in the wild (Culumber et al. in press). Even if we assume that our measures were of shoaling preferences, *a priori* predictions are not consistent with our data: (1) Melanistic spots could be confounded with black spot disease caused by a parasitic infection common in this system (Tobler et al. 2007, 2014), and both female types might avoid melanistic males as in *Gambusia* (Tobler & Schlupp 2008). Our behavioral data do not support this scenario. (2) Females do not attend to melanism even if spots do resemble parasites. Female *P. latipinna* do not appear to attend to male spots and shoal at random with spotted and unspotted males (Tobler et al. 2006). However, in this scenario, none of the female types should exhibit a preference. This again does not fit our data, because melanistic females exhibited a significant preference for spotted males. (3) Shoaling with a melanistic male could incur a cost if it attracts attention from a visual predator. In this scenario, melanistic females should prefer melanistic males. As melanistic females are already conspicuous, they could share the potential cost and increase their odds of survival by shoaling

with other melanistic individuals. However, non-melanistic females should avoid shoaling with melanistic fish in this case, because shoaling with a conspicuous individual would increase their probability of detection. This is particularly true in sulfur spring habitats where colloidal sulfur gives the water a white coloration that would reduce detection of non-melanistic fish, but would cause fish covered with melanistic spots to have high contrast against the background.

There are at least two reasons why polymorphism in preference for melanism could exist. The first explanation is that a mating preference for melanistic males may be adaptive or non-adaptive depending upon a female's phenotype. Melanistic phenotypes have been associated with stress resistance (Kittilsen et al. 2009), a variety of other physiological traits (Ducrest et al. 2008), and with reduced parasitism (Jacquin et al. 2011). However, predation against conspicuous color or pattern types often reduces the benefits acquired through sexual selection to males (Majerus et al. 2000; Hoekstra et al. 2005; Endler 1983). If the cost of associating with melanistic males differs between melanistic and non-melanistic females, this could generate variation in female preferences. Further work will be needed to disentangle the potential interaction of factors that may strike a balance between sexual and natural selection in this system, which could maintain polymorphism in female preferences.

Alternatively, preference in only the melanistic females may indicate a correlation or linkage between the preference and trait. Such associations between preferences and traits are known to contribute to assortative mating. For example, in *Heliconius* butterflies that mate assortatively based on wing pattern, there is tight genetic linkage between wing pattern and mate preference for wing pattern (Kronforst et al. 2006). Genetic correlations between songs and preference for songs that affect behavioral differences in crickets have also been shown to exhibit genetic linkage (Shaw & Lesnick 2009). Further work will be necessary to distinguish among these alternative hypotheses. Overall, our results complement recent findings demonstrating the role of non-random female preferences for body coloration between cave and surface-dwelling *P. mexicana* (Bierbach et al. 2013). Collectively, these studies are providing increasing evidence of the role of behavioral decisions related to female choice on the maintenance of color variation in the *P. mexicana* system. We should also point out that limits to migration due to natural selection as well as sexual selection against immigrants for

traits other than melanism could also contribute maintaining the near absence of melanism from the non-sulfidic site (Plath et al. 2013). This is especially true for the populations in this study located in the Pichucalco drainage, which is characterized by almost no gene flow and high reproductive isolation (Plath et al. 2013). This would suggest limited potential for effects of migration, and migrants would likely be further selected against by female aversion to immigrant males.

It is also important to note that there appears to be a balance preventing melanism from increasing in frequency in sulfidic populations. As melanistic females preferred melanistic males and other females exhibit random preference, it is expected that the melanistic phenotype would increase in frequency in sulfidic populations. It is therefore likely that there is a cost to melanism. A behavioral cost, such as that in *Gambusia* of increased aggression toward females (Horth 2003), seems unlikely given that melanistic females preferred melanistic males and non-melanistic did not exhibit avoidance. As melanistic individuals are likely visually conspicuous in the milky water of sulfide springs, melanism would likely increase detection by visual predators, which are present in the form of piscivorous birds (Riesch et al. 2010). Melanistic individuals have been documented to exhibit higher survival in predation experiments in at least one other poeciliid (Horth 2004), but most studies have found that conspicuous individuals pay a cost in the form of predation (Majerus et al. 2000; Hoekstra et al. 2005; Endler 1983). Although a variety of studies have demonstrated positive associations between melanism and stress resistance (but see Roulin et al. 2008), melanocortins stimulate metabolic rate and energy expenditure (Ducrest et al. 2008). Such physiological effects may be negative and especially important for sulfide spring fish, as they tend to have poor body condition as a consequence of resource acquisition constraints and increased costs of maintaining homeostasis in the toxic environment (Tobler 2008; Tobler et al. 2009a,b).

In summary, our study shows that female mate preferences between closely related populations can at once account for the maintenance of a conspicuous pigmentation polymorphism in one habitat type and contribute to the absence of the trait from adjacent habitats. Behavioral decisions associated with mate choice for melanism therefore provide a clear and strong mechanism explaining the maintenance of phenotypic variation. This adds a new example to an understudied but growing area of research on the behavioral and evolutionary ecology of pigmentation

and highlights the potential for this study system in this area of research. Finally, this study highlights the presence of intrapopulation variation in female mate preferences, and its potential role in driving morphological divergence.

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Ethical Standards

All experiments and animal care conformed to current laws and regulations in Mexico, where the work was conducted.

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