

Complementary effect of natural and sexual selection against immigrants maintains differentiation between locally adapted fish

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Abstract Adaptation to ecologically heterogeneous environments can drive speciation. But what mechanisms maintain reproductive isolation among locally adapted populations? Using poeciliid fishes in a system with naturally occurring toxic hydrogen sulfide, we show that (a) fish from non-sulfidic sites (*Poecilia mexicana*) show high mortality (95 %) after 24 h when exposed to the toxicant, while locally adapted fish from sulfidic sites (*Poecilia sulphuraria*) experience low mortality (13 %) when transferred to non-sulfidic water. (b) Mate choice

tests revealed that *P. mexicana* females exhibit a preference for conspecific males in non-sulfidic water, but not in sulfidic water, whereas *P. sulphuraria* females never showed a preference. Increased costs of mate choice in sulfidic, hypoxic water, and the lack of selection for reinforcement due to the low survival of *P. mexicana* may explain the absence of a preference in *P. sulphuraria* females. Taken together, our study may be the first to demonstrate independent—but complementary—effects of natural and sexual selection against immigrants maintaining differentiation between locally adapted fish populations.

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Introduction

An increasing body of literature documents adaptation's potential to drive genetic differentiation and ultimately speciation (e.g., Rundle et al. 2000; Nosil et al. 2002; Dettman et al. 2007; Langerhans et al. 2007), a phenomenon that has recently been termed 'isolation-by-adaptation' (Nosil et al. 2009). Of particular interest in the study of ecological speciation are the proximate mechanisms leading to and maintaining genetic differentiation among populations (Schluter 2001; Nosil et al. 2009). During ecological speciation, pre-zygotic isolation may arise when immigrants from foreign, ecologically divergent habitats are selected against (Hendry 2004; Nosil et al. 2005). This may occur through natural selection, if immigrants have reduced

viability (extrinsic reproductive isolation; e.g., Matute et al. 2009; Tobler et al. 2009a), or by sexual selection, if maladapted individuals are discriminated against during mate choice (e.g., Lorch et al. 2003).

In the present study, we examined the roles of natural and sexual selection against immigrants in a system of parapatrically occurring livebearing fishes (family Poeciliidae) at the Baños del Azufre near Teapa in Tabasco, Mexico, which are characterized by high concentrations of toxic hydrogen sulfide (H_2S ; Tobler et al. 2008a). No barriers other than water chemistry prevent fish movement in this system (Tobler et al. 2008a). Only two highly specialized fish species, *Poecilia sulphuraria* and *Gambusia eurystoma*, are found endemically in sulfidic habitats, whereas fish communities in non-sulfidic habitats are species-rich and include the related taxa *Poecilia mexicana* and *Gambusia sexradiata* (Tobler et al. 2008a). Unlike *P. mexicana* from non-sulfidic sites (Fig. 1a), *P. sulphuraria* are characterized by a large head and a correlated increase in total gill surface area (M. Tobler et al., unpublished data; Fig. 1b; see also Tobler et al. 2008b for enlarged heads in *P. mexicana* from another sulfidic system). This increase in head and gill size likely represents an adaptation to the hypoxic conditions in sulfidic environments. Furthermore, some *P. sulphuraria* exhibit conspicuous lip appendages (Alvarez del Villar 1948; Tobler and Plath 2009; Fig. 1b), which are thought to increase the efficiency of aquatic surface respiration (Winemiller 1989; Tobler et al. 2008a; Tobler and Plath 2011). Hence, morphological divergence in response to divergent ecologically-based natural selection clearly sets the stage for mate discrimination based upon morphological traits. Based on sequence divergence of mitochondrial genes, the two species appear to be closely related. *P. sulphuraria* essentially forms a monophyletic sister clade to a northern clade of *P. mexicana*, which together are sister taxa to a clade of *P. mexicana* from non-sulfidic habitats in southern Mexico (M. Tobler et al., unpublished data).

We focused on the two locally adapted *Poecilia* species and employed female mate choice experiments to test for pre-zygotic isolation through discrimination against immigrants, and reciprocal translocation experiments to test for differential viability between immigrant and resident fish.

Methods

Standard dichotomous mate choice experiments (association preference tests) were conducted on site in January 2010. Males of both species under study do not exhibit any courtship displays prior to gonopodial thrusting (Parzefall 1969; Plath 2008; Plath et al., unpublished data). Given that molly males constantly attempt to mate with females (e.g., even directly upon capture; Riesch et al. 2008), it is likely

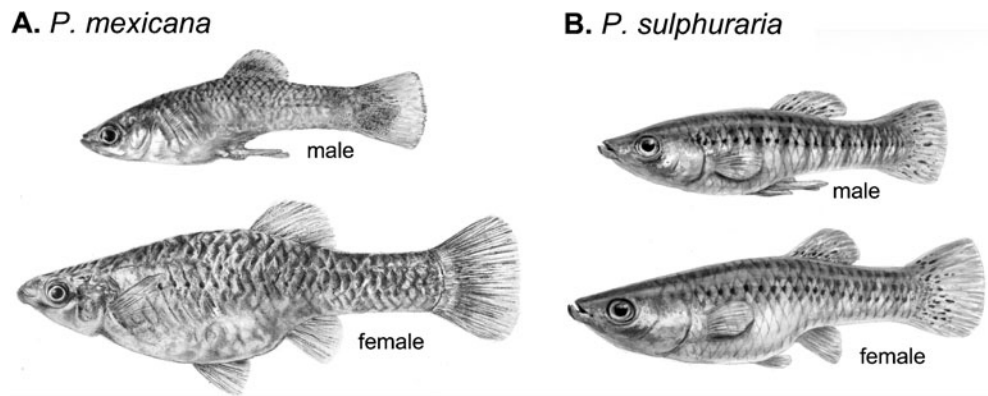
that time spent by a female associating with a given male (leading to physical proximity) facilitates male copulation attempts by that particular male. Also, wild-caught mollies readily express mating preferences directly upon capture (see Schlupp et al. 2010 for *Poecilia sphenops*). A recent study by Walling et al. (2010) experimentally demonstrated that association preferences actually do translate into male reproductive success in green swordtails, *Xiphophorus hellerii* (Poeciliidae), so association preferences should translate into more copulations with the preferred male also in *P. mexicana* and *P. sulphuraria*.

P. sulphuraria were collected in a sulfidic creek at the Baños del Azufre [between sites (1) and (2) in Tobler et al. (2008a)], while *P. mexicana* originated from a nearby freshwater site (Río Ixtapangajoya). Upon collection and prior to testing, test fish were brought to our temporary field laboratory at the Baños del Azufre and held overnight at ambient temperature in aerated black Sterilite® containers ($43 \times 31 \times 32$ cm), filled with water from the respective collection sites. None of the focal females gave birth before the tests, so most females were probably gravid.

Mate choice tests were conducted in three identical portable test tanks ($42.6 \times 30 \times 16.5$ cm) built from UV transparent Plexiglas. Each tank was visually divided into three equally-sized zones by black marks on the outside. The central zone was designated the neutral zone, the two lateral zones as preference zones. Two stimulus males were presented in two smaller tanks ($19.5 \times 30 \times 14.5$ cm) on either side of the test tank. Hence, the focal female could choose on the basis of visual cues potentially including morphological (see “Introduction” section; Fig. 1) and behavioral differences between the stimulus males. Before each trial, a stimulus male was placed into each side tank. Then, a female was introduced into the test tank. The choice tanks we used are relatively small, so that females were able to see both stimuli at all times. Test fish would typically freeze on the bottom of the test tank for few minutes after they were introduced, so a trial began only after the focal female had started to swim freely in the water column. We measured the time the female spent in each preference zone during a 5-min observation period. To detect side biases, the stimuli were switched immediately after the first trial and measurement was repeated. Once a trial was completed, all fish involved were measured for standard length (SL).

Four different experiments were conducted, which reflect all possible migration events between adjacent habitat types: (1) *P. sulphuraria* ($N=23$) and (2) *P. mexicana* females ($N=23$) were tested with con- and heterospecific stimulus males in non-sulfidic water that was taken from a spring source at the Baños del Azufre; (3) *P. sulphuraria* ($N=24$) and (4) *P. mexicana* females ($N=23$) were also tested in sulfidic water taken directly from the sulfidic creek. All fish (focal females

Fig. 1 a Drawings of a male (above) and female *P. mexicana* (below) collected in a non-sulfidic site in southern Mexico (drawings by M. Ziege). **b** Drawings of a male (above) and a female *P. sulphuraria* (below) from the Baños del Azufre (drawings by D. Caspari). Note the conspicuous lip appendages and the relatively larger head size in **b**



and stimulus males) were used in only one test after which they were released into the respective collection site.

For the mate choice experiment, we summed association times near con- and heterospecific males from both test parts (before and after switching of side-assignments) and tested for female preferences within each test situation by comparing individual strength of preference (SOP)-values against a random distribution of SOP=0 using one-sample *t*-tests. The strength of female preference was calculated as $[(\text{time spent near conspecific male} - \text{time near heterospecific male}) / (\text{time near conspecific male} + \text{time near heterospecific male})]$, so SOP values could range from -1 (complete avoidance of the conspecific male) to $+1$ (complete preference for the conspecific male). To test for differences among species and treatments, SOP values were used as the dependent variable in a fully factorial general linear model (GLM), in which ‘focal female species’ and ‘treatment’ (sulfidic or non-sulfidic water) were included as factors. ‘Body size difference’ between the two stimulus males was included as a covariate because males were not always perfectly size-matched due to limitations under field conditions (see online supplemental data, Table S1).

The translocation experiment was conducted in August 2008; *P. mexicana* used for this experiment came from a clearwater tributary that merges with the sulfidic creek at the Baños del Azufre [site (10) in Tobler et al. (2008a)]. We performed reciprocal translocation experiments using 20-L buckets as experimental containers. Ten buckets were placed in shallow areas both in sulfidic and non-sulfidic water, and the experiment was conducted in two runs (for a total $N=40$). The two sites were located about 250 m above the confluence of the non-sulfidic and sulfidic creeks. At both sites, water was shallow with moderate flow, and the substrate consisted of gravel and rocks (see online supplement, Fig. S1). In order to maintain constant exchange of water with the environment, two holes (18×32 cm) were cut on opposite sides of the buckets and then sealed with 1.5-mm plastic mesh. Bucket lids were perforated with ~50 small holes to allow for air exchange. Experimental containers were then placed directly into a shallow area of the natural

habitats, and we added a 3.5 cm layer of natural substrate. Prior to the experiments, water quality was measured in six randomly chosen buckets per habitat as well as outside of the buckets (see online supplemental data, Table S2).

Upon collection, fish were kept in insulated coolers. At the start of the experiment, six individuals from a given site were introduced into an experimental bucket. In another sulfidic system (the Cueva del Azufre system), *P. mexicana* males are known to be more susceptible to the negative effects of H_2S than females (Plath et al. 2007). However, for the present study, only females were used, so any effect due to sex differences can be ruled out. Half of the buckets at each site were set up with resident fish, half with fish from the other habitat type. We assessed mortality after 24 h. All fish were measured for SL, and surviving individuals were released at their original collection site.

For data analysis, each bucket was treated as an independent replicate. Arcsine-transformed survival rates (proportions of surviving individuals per bucket) were analyzed using a GLM with ‘population of origin’ and ‘experimental location’ as independent variables. We approximated effect strengths using partial eta squared (η_p^2). All statistical analyses were performed using SPSS 17 (SPSS Inc., Chicago, IL, USA).

Results

Sexual selection

P. mexicana females showed a strong preference for conspecific males in clear, but not in sulfidic water. *P. sulphuraria* females, on the other hand, did not exhibit a preference in either treatment (Fig. 2). This difference between species was reflected in a significant effect of ‘species×treatment’ in the GLM (Table 1).

Natural selection

‘Habitat’ ($F_1=82.6$, $P<0.001$, $\eta_p^2=0.697$), ‘species’ ($F_1=95.6$, $P<0.001$, $\eta_p^2=0.726$), and their interaction term ($F_1=$

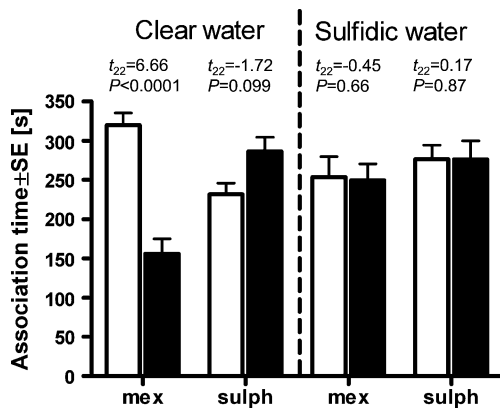


Fig. 2 Mean (\pm SE) association times of females with a conspecific male (white bars) and a heterospecific male (black bars). *mex*, *P. mexicana*; *sulph*, *P. sulphuraria*. One sample *t* tests were used to compare the strength of preference (SOP) for the conspecific stimulus male against a random distribution (SOP=0)

183.2, $P < 0.001$, $\eta_p^2 = 0.836$) had a significant effect on survival of the test fish. While *P. sulphuraria* survived almost equally well in sulfidic and non-sulfidic water, *P. mexicana* had reduced survival in the sulfidic habitat (Fig. 3).

Discussion

Our experiments demonstrate that natural and sexual selection against immigrants in our study system act asymmetrically; each selective force appears to be reducing gene flow across habitats only in one direction. However, since they are complementary in action, they may nonetheless effectively restrict gene flow between the two locally adapted *Poecilia* populations. Reduced gene flow from non-sulfidic to sulfidic habitats is mediated by natural selection, and sulfide-induced mortality of non-adapted fish is straightforward to explain by the lack of specific adaptations to cope with sulfide toxicity and hypoxic conditions (Tobler et al. 2008a; Peters et al. 1973;

Table 1 Results from a fully factorial GLM using the strength of preference (SOP) for the conspecific male as dependent variable, ‘species’ (*P. mexicana* or *P. sulphuraria* focal females) and ‘treatment’ (sulfidic or non-sulfidic water) as factors, and ‘body size difference’ (i.e., between the two stimulus males) as a covariate

Effect	df	Mean square	<i>F</i>	<i>P</i>	η_p^2
Body size difference	1	0.080	3.05	0.084	0.033
Species	1	0.036	1.38	0.24	0.015
Treatment	1	0.257	9.85	0.002	0.101
Species x treatment	1	0.344	13.19	<0.0001	0.130
Error	88	0.026			

Significant effects are in bold typeface

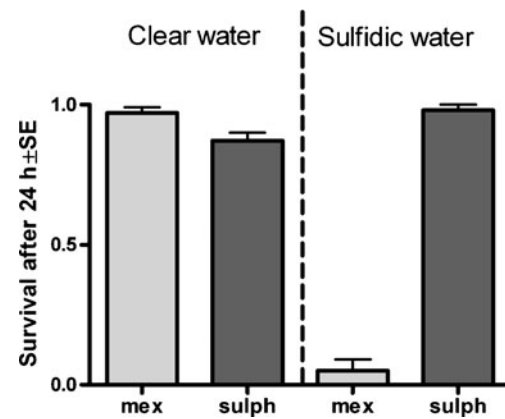


Fig. 3 Mean (\pm SE) percent survival of *P. mexicana* (*mex*, light gray bars) and *P. sulphuraria* (*sulph*, dark gray bars) in reciprocal translocation experiments between clear water and sulfidic habitats after 24 h ($N=10$ per combination). Each replicate consisted of six individual fish being kept in 20-l confinements

reanalyzed in Plath and Tobler 2010; Plath et al. 2010). In contrast, there is no sexual selection against immigrants in sulfidic habitats, as *P. sulphuraria* females did not exhibit mating preferences under any experimental condition. There are several potential reasons for the absence of assortative mating in sulfidic habitats. First, selection for reinforcement, i.e., direct selection on the evolution of mating preferences to avoid the production of unfit hybrids, is not expected in *P. sulphuraria*, since natural selection against migrants from non-sulfidic to sulfidic habitats is strong. Second, survival in toxic hydrogen sulfide is costly even for locally adapted individuals (Plath et al. 2007; Tobler 2008; Tobler et al. 2009b), and accordingly, costly behaviors are reduced in *P. mexicana* from another sulfidic system (e.g., Plath 2008). Therefore, choosiness may be a trait that poeciliids in toxicity generally cannot afford to express (compare Wong and Jennions 2003). Also, *P. mexicana* from non-sulfidic habitats did not express a mating preference under sulfidic conditions. In this case, the lack of preference is likely related to the direct toxic effects of sulfide. In our experiments, many *P. mexicana* females appeared lethargic when tested in sulfidic water, but these behavioral changes were not quantified.

In contrast, the reduction of gene flow from sulfidic into non-sulfidic habitats appears to be mediated by sexual selection, as *P. sulphuraria* do not exhibit drastic reductions in survival when transferred into non-sulfidic waters. Instead, *P. mexicana* females show strong assortative mating under non-sulfidic conditions, which likely reflects reinforcement in response to the presence of locally occurring related species (Rundle and Schluter 1998; Magurran and Ramnarine 2005). *P. mexicana* should be under strong selection to discriminate against immigrant *P. sulphuraria* males if hybrids have a selective disadvantage, e.g., because of maladaptive expression of morphological,

physiological, or life history traits (e.g., Riesch et al. 2010a, b; Plath et al. 2010 for discussion). Even though we detected low short-term mortality in translocated *P. sulphuraria*, sublethal effects are likely to occur. In another system with presence of H₂S (the Cueva del Azufre system), fish from sulfidic habitats experience a strong fitness reduction (and high mortality) when transferred into non-sulfidic water (Tobler et al. 2009a). We can only speculate at this point that hybrids in the long run could show reduced performance in either habitat type, and competition with better adapted (non-hybrid) individuals, as well as selection against maladapted individuals through predation (Tobler 2009) may be operating.

At this point, the incidence of hybridization and the extend of gene flow between *P. sulphuraria* and *P. mexicana* are unknown. Preliminary analyses of mitochondrial DNA sequences did not reveal any evidence for introgression, as *P. mexicana* in habitats adjacent to the sulfidic spring are part of a more distantly related clade (Tobler et al., unpublished data); still, more (nuclear) markers will need to be analyzed to unequivocally answer this question. Based on morphological analyses, we have identified some specimens with an intermediate phenotype between the two species. However, since morphological variability is high particularly in *P. mexicana*, it is unclear whether these intermediate individuals are actually of hybrid origin or simply represent extreme phenotypes within the “normal” variation of *P. mexicana* (M. Tobler, unpublished data). This indicates the need for further studies incorporating thorough population genetic analyses to test for patterns of gene flow across habitat types in this system. Data from another sulfidic system, where *P. mexicana* have colonized sulfidic springs and have evolved into distinct ecotypes, indicate that reproductive isolation is strong and gene flow between habitats minimal despite spatial proximity and the lack of physical barriers (Tobler et al. 2008b; Plath et al. 2010).

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