

Natural and sexual selection against immigrants maintains differentiation among micro-allopatric populations

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Abstract

Local adaptation to divergent environmental conditions can promote population genetic differentiation even in the absence of geographic barriers and hence lead to speciation. But what mechanisms contribute to reproductive isolation among diverging populations? We tested for natural and sexual selection against immigrants in a fish species inhabiting (and adapting to) nonsulphidic surface habitats, sulphidic surface habitats and a sulphidic cave. Gene flow is strong among sample sites situated within the same habitat type, but low among divergent habitat types. Our results indicate that females of both sulphidic populations discriminate against immigrant males during mate choice. Furthermore, using reciprocal translocation experiments, we document natural selection against migrants between nonsulphidic and sulphidic habitats, whereas migrants between sulphidic cave and surface habitats did not exhibit increased mortality within the same time period. Consequently, both natural and sexual selection may contribute to isolation among parapatric populations, and selection against immigrants may be a powerful mechanism facilitating speciation among locally adapted populations even over very small spatial distances.

Introduction

Unravelling the mechanisms underlying the origin of biodiversity is a pivotal goal in evolutionary biology. Divergent selection cannot only lead to adaptive trait divergence (Kawecki & Ebert, 2004), but also reproductive isolation among populations and thus (ecological) speciation (Schluter, 2000; Rundle & Nosil, 2005). An increasing body of literature documents adaptation's potential to drive genetic differentiation among populations in the wild as well as in the laboratory (e.g. Funk, 1998; Rundle *et al.*, 2000; Nosil *et al.*, 2002; Dettman *et al.*, 2007; Langerhans *et al.*, 2007), a phenomenon termed 'isolation-by-adaptation' (Nosil *et al.*, 2009a). However, the proximate mechanisms of ecological

speciation, i.e. the question of how exactly adaptation translates into reproductive isolation, are often less well understood (Nosil *et al.*, 2009a,b). Post-zygotic reproductive isolation among allopatric populations can arise over time when populations diverge genetically (e.g. Ludlow & Magurran, 2006). During ecological speciation, however, prezygotic isolation may arise when immigrants from foreign, ecologically divergent habitats are selected against (Hendry, 2004; Nosil *et al.*, 2005). This may occur by natural selection, if immigrants have a reduced viability (extrinsic reproductive isolation; e.g. Matute *et al.*, 2009), or by sexual selection, if poorly adapted individuals are discriminated against during mate choice (Lorch *et al.*, 2003; Snowberg & Benkman, 2007, 2009).

We investigated potential mechanisms contributing to reproductive isolation in a small livebearing fish (*Poecilia mexicana*) occurring along a gradient of abiotic environmental conditions. Fish not only occur in normal surface creeks, but also in a toxic, hydrogen sulphide-containing surface creek, and a sulphidic cave (Tobler *et al.*, 2006).

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No major physical barriers prevent fish from moving between different habitat types that are only a few hundred metres apart. Still, divergent environmental conditions have been shown to drive adaptive divergence in a set of behavioural, life history, morphological and physiological traits (Parzefall, 2001; Plath *et al.*, 2007a; Tobler *et al.*, 2008a; Riesch *et al.*, 2009). Although all locally adapted forms can be crossbred in the laboratory (Parzefall, 1979), remarkably strong genetic differentiation over small geographic distances has been uncovered (Plath *et al.*, 2007a; Tobler *et al.*, 2008a). While gene flow is high within the same habitat type, it is virtually zero among different habitat types (see Figure S1 for a re-analysis of microsatellite data previously published in Tobler *et al.*, 2008a). Here, we tested for potential natural and sexual selection against immigrants. We employed female mate choice experiments to test whether resident males are preferred over immigrants, and used reciprocal translocation experiments to test for differential viability between immigrant and resident fish.

Materials and methods

Study system

All experiments were conducted between August and October 2008 in the Cueva del Azufre system (N 17.442°, W 92.775°) near Tapijulapa (Tabasco, Mexico). Fish were collected in an illuminated front chamber of the sulphidic cave (Cueva del Azufre), a sulphidic surface creek (El Azufre) and nonsulphidic surface habitats. Detailed information on the structure and the water chemistry of these habitats can be found in Tobler *et al.* (2006, 2008a). As the three habitat types are arranged in a linear order, i.e. water flows from the cave to the El Azufre and then to the nonsulphidic surface habitats, we focussed all experiments on the two potential contact zones. Hence, we contrasted fish from the sulphidic cave to fish from the sulphidic surface habitat and fish from the sulphidic surface habitat to fish from nonsulphidic surface habitats (different nonsulphidic surface habitats were used for the mate choice and the translocation experiment: Arroyo Bonita fish were used for the sexual selection experiment and Arroyo Cristal fish for the natural selection experiment).

Sexual selection

We tested if females would exhibit a preference for males from their own population over males from a different population using a standard dichotomous mate choice test (Ryan *et al.*, 1996; Houde, 1997; Plath *et al.*, 2004; Wong *et al.*, 2005). After collection and prior to testing, all test fish were brought to a nearby field station and acclimated for 24 h in closed and aerated black Sterilite® containers (43 × 31 × 32 cm, length × width × height) filled with water from the respective collection sites.

Males and females were kept separately. Tests were conducted in five identical test tanks (43 × 17 × 30 cm), which were built with UV-transparent Plexiglas. Each tank was divided into three equal zones: a central neutral zone and the two lateral preference zones. The stimuli were presented in two smaller tanks (20 × 15 × 30 cm) on either side of the test tanks. As focus and stimulus fish were in separate containers, we were able to keep them in water from their respective collection sites during the experiments. Before each trial, stimulus males were placed into the side tanks (one male per side). Then a female was added, and to allow for acclimation, the trial was started once the female started to swim freely. 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 the measurement was repeated. The actual choice test thus consisted of two observation periods that lasted for 10 min.

In total, four different experiments were conducted, which reflect all possible migration events between adjacent habitat types: (1) females from the sulphidic cave were tested with males from their own population and males from the adjacent El Azufre; (2) El Azufre females were tested with males from their own population and males from the cave as well as (3) males from the nonsulphidic Arroyo Bonita and (4) Arroyo Bonita females were tested with males from their own population and El Azufre males.

Natural selection against immigrants

We performed reciprocal translocation experiments between nonsulphidic and sulphidic surface habitats as well as between sulphidic surface and cave habitats using 20-L buckets as experimental containers. To maintain constant exchange of water with the environment, two holes (18 × 32 cm) were cut on opposite sites 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 equipped with 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 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. Half of the buckets at each site were set up with resident fish, half with fish from the other habitat type. The experiment was originally designed to measure long-term changes in body condition. As buckets were not put in the immediate spring areas but downstream, where H₂S concentrations were comparatively low [a range of 9–42 µM was measured during multiple visits from 2004 to 2008, which contrasts to concentrations peaking

well over 300 μM closer to sulphidic springs (Tobler *et al.*, 2006, 2008a)], even fish from nonsulphidic habitats were expected to survive for longer periods. However, high mortalities forced us to use survival after 24 h as a dependent variable. All experiments were immediately terminated after this period, fish were measured for standard length (SL), and surviving individuals were released at their original collection site.

Statistical analyses

In the mate choice experiment, the times females spent with each of the males were used as dependent variables in an *rMANCOVA*. 'Experiment' was used as an independent variable to test for differences in female preferences among populations. Also, due to systematic size differences among populations (Plath, 2008; Tobler *et al.*, 2008b; Tobler, 2009), it was not always possible to perfectly size-match the two stimulus males (SL of males from same population as female, mean \pm SD: 31.3 \pm 6.5 mm; SL of males from different population as female: 30.0 \pm 5.9 mm; also see Table S1 for descriptive statistics on size of fish used in all experiments); hence, we included 'male size difference' (resident-foreign population) as a covariate in the analysis.

In the translocation experiment, each bucket was treated as an independent replicate. Arcsine square root-transformed survival rates (proportions of surviving individuals per bucket) were analysed using general linear models 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 16* (SPSS Inc., Chicago, IL, USA).

Results

Sexual selection

Female mate choice experiments revealed significant preferences for males from the same population over males from an ecologically divergent population (see significant within subject effect in Table 1). Even though this effect did not differ significantly among populations ('male type by experiment' in Table 1), females from the nonsulphidic surface habitat qualitatively showed a weaker preference than the other populations (Fig. 1). A significant between-subjects effect of the factor 'experiment' indicates that females overall differed in total time spent associating with the stimulus males (Table 1).

Natural selection

Translocating fish between nonsulphidic and sulphidic surface habitats revealed a significant interaction between 'population of origin' and 'experimental location' ($F_1 = 128.252$, $P < 0.001$, $\eta_p^2 = 0.848$); i.e. fish

Table 1 Results from the mate choice experiment (*rMANCOVA*).

Source	d.f.	Mean square	<i>F</i>	<i>P</i>	η_p^2
Within-subjects effects					
rm (male type)	1	133 235.338	4.487	0.037	0.056
rm \times Δ SL	1	15 991.576	0.539	0.465	0.007
rm \times Experiment	3	33 712.689	1.135	0.340	0.043
Error	75	29 695.545			
Between-subjects effects					
Δ SL	1	784.509	0.341	0.561	0.005
Experiment	3	6368.378	2.770	0.047	0.100
Error	75	2299.218			

Association times near resident and immigrant males (repeated measurements, rm) were used as the dependent variables. Significant *P*-values are given in bold.

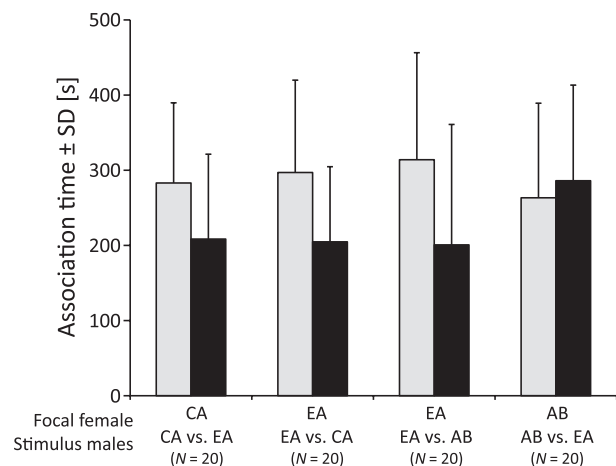


Fig. 1 Mean (\pm SD) association times of females with a male of the same population (light grey bars) and a male from an adjacent but ecologically divergent habitat (dark grey bars). CA: Cueva del Azufre (sulphidic cave); EA: El Azufre (sulphidic surface habitat); AB: Arroyo Bonita (nonsulphidic surface habitat).

from nonsulphidic habitats had a low survival in sulphidic habitats, whereas fish from sulphidic habitats performed poorly under nonsulphidic conditions (Fig. 2a). Neither the factor 'population of origin' ($F_1 = 0.859$, $P = 0.364$, $\eta_p^2 = 0.036$) nor 'experimental location' ($F_1 = 1.103$, $P = 0.305$, $\eta_p^2 = 0.046$) *per se* was significant. Measurements of water quality indicated no differences between buckets and the respective surrounding environment (Table S2). However, sulphidic habitats differed from nonsulphidic habitats not only in the presence and absence of H_2S , but also in higher temperature and specific conductivity as well as lower pH and dissolved oxygen concentration.

Translocations between sulphidic surface and cave habitats revealed no significant mortality within 24 h (Fig. 2b). None of the factors included in the analysis nor their interaction had a significant effect ('population of

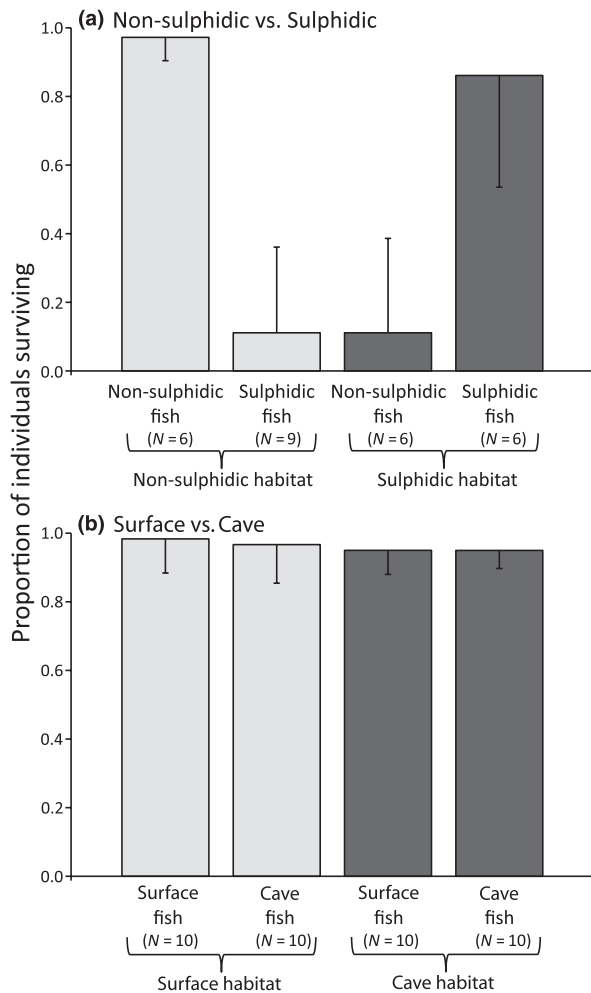


Fig. 2 Mean (\pm SD) survival of *Poecilia mexicana* in translocation experiments between nonsulphidic and sulphidic surface habitats (a) and sulphidic cave and surface habitats (b). Each replicate consisted of six individual fish that were kept in 20-L confinements.

origin': $F_1 = 0.095$, $P = 0.760$, $\eta_p^2 = 0.003$; 'experimental location': $F_1 = 0.402$, $P = 0.530$, $\eta_p^2 = 0.011$; 'population of origin \times experimental location': $F_1 = 0.156$, $P = 0.696$, $\eta_p^2 = 0.004$). The measured water parameters did not differ between surface and cave habitats (Table S2).

Discussion

Selection against immigrants from foreign, ecologically divergent habitats, i.e. the phenomenon that specific adaptations to one environment may reduce performance in another one, is one of the simplest forms of reproductive isolation. Our mate choice and translocation experiments indicated that sexual as well as natural selection against immigrants indeed contribute to the genetic divergence of fish living in different habitat types

of the Cueva del Azufre system. Here, fish differ phenotypically among different habitat types; e.g. fish from habitats containing hydrogen sulphide exhibit larger heads and gills than conspecifics from nonsulphidic habitats, and fish from cave habitats have reduced eye size and pigmentation but more elaborated nonvisual senses compared to conspecifics from surface habitats (Parzefall, 2001; Tobler *et al.*, 2008a).

In the mate choice trials, females discriminated against males from foreign habitats and preferred to associate with males from their own habitat type. Immigrant males from ecologically divergent habitats are consequently at a disadvantage by sexual selection (see also Svensson *et al.*, 2006; Grant & Grant, 2008). A similar result has been documented in mosquitofish (*Gambusia hubbsi*) from different predator environments (Langerhans *et al.*, 2007). Because of geographical isolation of different *Gambusia* populations, reproductive isolation in the form of female mate choice in these mosquitofish has been hypothesized to have evolved as a by-product of natural selection on morphological traits (Langerhans *et al.*, 2007). In the case documented here, however, we can only speculate about the mechanisms giving rise to the observed female preference. Potential mechanisms range from direct natural selection on premating isolation (i.e. reinforcement, Schluter, 2001; Rodriguez *et al.*, 2004) to learned preferences for resident phenotypes (Verzijden & ten Cate, 2007). In either case, the consequence is that even in the absence of survival effects on individuals migrating among habitats their fitness is reduced by sexual selection.

Translocation experiments indicated that viability of fish is eminently low when transferred between sulphidic and nonsulphidic habitats. Low viability in fish translocated from nonsulphidic to sulphidic habitats was not surprising; hydrogen sulphide is a potent toxicant lethal to most metazoans as it blocks cell respiration (Bagarinao, 1992; Grieshaber & Völkel, 1998). *Poecilia mexicana* living in sulphidic environments have evolved physiological pathways to detoxify sulphide (Peters *et al.*, 1973) and respiratory adaptations such as enlarged gills (Plath *et al.*, 2007b; Tobler *et al.*, 2008a) that mediate sulphide tolerance. Respiratory adaptations are especially relevant in sulphidic systems, as the presence of sulphide is strongly correlated with hypoxia (Chen & Morris, 1972; Tobler *et al.*, 2006), and at the same time, oxygen is required in the physiological detoxification of sulphide (Curtis *et al.*, 1972; Bagarinao, 1992). Consequently, nonadapted individuals likely succumb to the toxic effects of H_2S .

Why fish transferred from a toxic to a nontoxic habitat experience equally low viability is less straightforward to explain. Surely, fish from sulphidic habitats do not require H_2S to thrive, however, sulphidic and nonsulphidic habitats also differ in other aspects of water quality, specifically lower temperature and specific conductivity as well as higher pH and oxygen concentra-

tions. Yet all values are well within the range of what *P. mexicana* and congeneric species experience throughout their ranges. Other species of *Poecilia* have even been documented to tolerate much higher fluctuations in such water quality parameters (e.g. Schlupp *et al.*, 2002; Nordlie, 2006). We hypothesize that high mortalities of fish from sulphidic habitats in nonsulphidic environments are caused by oxidative stress. Oxygen is inherently toxic due to its biotransformation into reactive oxygen species, and organisms have evolved biochemical pathways with antioxidant activity (e.g. superoxide dismutase, catalase, and glutathione systems, Halliwell & Gutteridge, 1999). During hypoxia, the expression of antioxidant enzymes is often down-regulated (Hermes-Lima & Zenteno-Savin, 2002; Olsvik *et al.*, 2006), so that subsequent exposure to normoxic conditions causes substantial oxidative stress with profound fitness consequences (Sies, 1986; Hermes-Lima & Zenteno-Savin, 2002). Such oxidative stress, maybe in combination with the often poor body condition and energy limitation of fish from sulfidic habitats (Plath *et al.*, 2007b; Tobler, 2008), may explain the mortality observed in our experiment.

A major open question remains whether acclimatization over prolonged periods of time would allow migrants to cope with the changed environmental conditions. There is ample evidence for transcriptional regulation of antioxidant proteins depending on ambient oxygen concentrations (e.g. Cooper *et al.*, 2002; Zenteno-Savin *et al.*, 2006), and *P. mexicana* from sulphidic habitats appear to be able to adjust to higher oxygen concentrations as evidenced by the fact that we can keep and breed populations from sulphidic habitats in the laboratory under normoxic conditions. In contrast, little is known about organisms' responses to repeated exposure to sulphide. Most organisms likely are able to detoxify H₂S to some extent, as sulphide is present in very low concentrations in the atmosphere and also produced endogenously by eukaryotic cells as a product of catabolism of cysteine (Kimura, 2002). Furthermore, an enzyme that plays a key role in sulphide homeostasis, sulphide-quinone oxidoreductase (Marcia *et al.*, 2009), has recently been found in all domains of life except for plants (Shahak & Hauska, 2008). Nonetheless, only few organisms are able to withstand continued exposure to higher concentrations of H₂S (Bagarinao, 1992). Experiments using *P. mexicana* by Peters *et al.* (1973) indicated that even fish from nonsulphidic habitats could cope with H₂S temporarily but for significantly shorter time periods than conspecifics from sulphidic habitats (reanalysed in Plath & Tobler, in press). Consequently, the strength of selection especially against fish moving from sulphidic to nonsulphidic habitats but also *vice versa* may be lower than that found in our translocation experiments. Laboratory experiments that manipulate sulphide and oxygen concentrations more subtly as well as a more detailed knowledge about the underlying physiological

and biochemical mechanisms will be required to properly address these aspects in the future.

Contrary to translocations between sulphidic and nonsulphidic habitats, a transfer of fish between sulphidic cave and surface habitats had no effect on survival in either direction. This is not unexpected; the presence or absence of light is unlikely to affect survival within only 24 h. Nonetheless, cave and surface populations differentiated phenotypically and differ primarily in the expression of pigmentation and sensory structures (Gordon & Rosen, 1962; Parzefall, 2001). Cavefish are characterized by having reduced eyes (Plath *et al.*, 2007a; Tobler *et al.*, 2008a), but hypertrophied gustatory and lateral line organs (Walters & Walters, 1965; Parzefall, 1970). The presence and absence of light thus directly affects the relative competitive ability of cave and surface fish. For example, similar translocation experiments that involved the presence of a predator (a giant water-bug of the genus *Belostoma*) indicated direct selection against immigrants from a divergent habitat type as bugs preferentially attacked cavefish in light but surface fish within the cave (Tobler, 2009). Furthermore, darkness is associated with strong shifts in life-history strategies, namely, reduced fecundity and increased offspring size in the cave form, which may be maladaptive in an ecologically divergent environment (Riesch *et al.*, 2009).

Overall, we find low rates of gene flow between sulphidic surface and cave habitats but none between nonsulphidic and sulphidic surface habitats, which is congruent with the results from our natural selection experiments. In contrast, sexual selection probably plays a comparatively smaller role in mediating reproductive isolation, as matings between fish from different populations may occur despite the presence of mating preferences. In conclusion, there is mounting evidence that selection against immigrants contributes substantially to reproductive isolation among adjacent populations living in different habitat types. Particularly, reduced immigrant viability – either through selection by the abiotic environmental factor (H₂S in sulphidic vs. nonsulphidic habitats) or through a combination of abiotic and biotic factors (light and predators in cave vs. surface habitats, Tobler, 2009) – appears to contribute to maintaining genetic differentiation across small spatial scales.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Table S1 Sample sizes and standard lengths (\pm standard deviation) of fish used in mate choice experiments.

Table S2 Measurements of temperature, pH, specific conductivity, dissolved oxygen and H₂S concentrations at all sites used in the reciprocal translocation experiments.

Figure S1 First generation migrants as calculated by GENECLASS2.

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Online Supplementary Material**Natural and sexual selection against immigrants maintains differentiation among micro-allopatric populations**

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Migration patterns within and between ecologically different habitats

To demonstrate restricted gene-flow between *P. mexicana* populations from adjoining, but ecologically different habitat types within the Cueva del Azufre system (see Table S2 for water chemistry measurements), a total of $N = 240$ individuals were genotyped using ten unlinked nuclear microsatellite loci (Tiedemann et al., 2005; Plath et al., 2007). Allele fragment length data were re-analyzed from a previously published study (Tobler et al., 2008). Included in this analysis were several representative non-sulphidic surface sites in the vicinity of the Cueva del Azufre (Arroyo Bonita: $N = 24$; Arroyo Cristal: $N = 20$; Arroyo Tres: $N = 19$; Río Amatan: $N = 24$; Río Oxolotán: $N = 24$), two sites along the sulphidic surface creek El Azufre (EAI: $N = 40$; EAII: $N = 20$), and samples from four cave chambers of the sulphidic Cueva del Azufre (chamber V: $N = 10$; X: $N = 20$; XI: $N = 19$; XIII: $N = 21$). To calculate first-generation migrants we employed GENECLASS2 (Piry et al., 2004), using the L_{home} likelihood computation, the Bayesian method of classification (Rannala & Mountain, 1997), and a threshold P -value of 0.05.

Figure S1 depicts numbers for first-generation migrants/dispersers as a proportion of the recipient population. Migration/dispersal events were common within the same habitat type, e.g., among the various non-sulphidic surface sites. Notably, not a single migration/dispersal event was detected between non-sulphidic and sulphidic habitats. Between the Cueva del Azufre (sulphidic cave) and El Azufre (sulphidic surface) some unidirectional migration/dispersal events were found; i.e., migration was always from inside the cave toward the surface habitat.

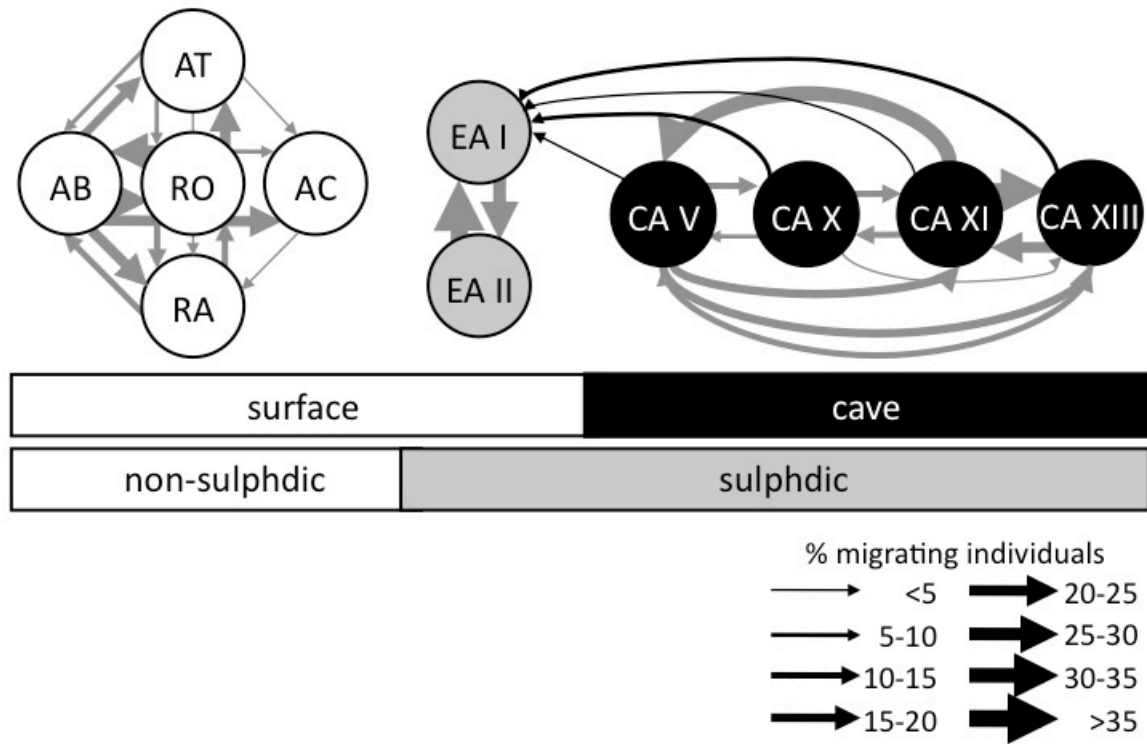


Figure S1. First generation migrants as calculated by GENECLASS2. Numbers of migrants are given as a proportion of the number of individuals in the recipient population, whereby the direction of the arrows indicates the direction of migration events, and arrow width indicates the fraction of migrants. Grey arrows represent migration events among sites of the same habitat type, black arrows among sites of divergent habitat types. White circles represent different collection sites in non-sulphidic surface habitats (AT: Arroyo Tres; AB: Arroyo Bonita; RO: Rio Oxolotan; AC: Arroyo Cristal; RA: Rio Amatan), grey circles represent sulphidic surface habitats (EA I: El Azufre I at cave resurgence; EA II: El Azufre II at big springs), and black circles represent different cave chambers within the Cueva del Azufre (CA).

Size of test fish

Table S1. Sample sizes and standard lengths (\pm standard deviation) of fish used in mate choice experiments.

	<i>N</i>	Standard length [mm]
Mate choice experiments		
<i>(1) CA female, CA vs. EA males</i>		
Focal females	20	26.5 \pm 6.0
Stimulus males CA	20	29.8 \pm 4.1
Stimulus males EA	20	29.1 \pm 2.1
<i>(2) EA female, EA vs. CA males</i>		
Focal females	20	30.8 \pm 8.1
Stimulus males EA	20	29.4 \pm 5.5
Stimulus males CA	20	28.4 \pm 5.0
<i>(3) EA female, EA vs. AB males</i>		
Focal females	20	32.8 \pm 4.9
Stimulus males EA	20	33.6 \pm 5.9
Stimulus males AB	20	31.0 \pm 8.1
<i>(4) AB female, AB vs. EA males</i>		
Focal females	20	31.7 \pm 10.9
Stimulus males AB	20	32.5 \pm 8.9
Stimulus males EA	20	31.7 \pm 6.5
Translocation experiments		
<i>(1) Non-sulfidic vs. sulfidic surface habitats</i>		
Non-sulfidic fish in non-sulfidic habitat	36	36.1 \pm 5.6
Sulfidic fish in non-sulfidic habitat	54	31.1 \pm 4.3
Sulfidic fish in sulfidic habitat	36	31.3 \pm 3.9
Non-sulfidic fish in sulfidic habitat	36	35.3 \pm 5.2
<i>(2) Sulfidic surface habitat vs. sulfidic cave</i>		
Surface fish in surface habitat	60	36.3 \pm 6.0
Cave fish in surface habitat	60	31.0 \pm 4.2
Cave fish in cave habitat	60	32.2 \pm 4.6
Surface fish in cave habitat	60	34.9 \pm 6.1

Water chemistry

Habitats in the Cueva del Azufre system are characterized by a peculiar water chemistry. Specifically, some habitats in the Cueva del Azufre system exhibit high concentrations of hydrogen sulphide (Tobler et al., 2006; 2008), which is a potent respiratory toxicant (Bagarinao, 1992; Grieshaber & Völkel, 1998). Prior to the start of the experiments, we measured water quality at all sites used in the reciprocal translocation experiments within as well as outside of the experimental enclosures. Temperature, specific conductivity, pH, and oxygen content were measured using a YSI multi-probe. For the measurement of H₂S concentrations, 1 ml of water was injected into a vial containing 1 ml of zinc acetate (0.12 M with 0.5 ml NaOH 1.5 M in a N₂-atmosphere) using a syringe. The vials were stored at room temperature, and photometric measurements were conducted in the laboratory according to Cline (1969). Water chemistry differed strongly between the sulfidic and the non-sulfidic habitat in all parameters measured (See table S2), but not between the cave and the surface habitat. Also, conditions within the experimental containers overall did not deviate from conditions outside.

Table S2. Measurements of temperature, pH, specific conductivity, dissolved oxygen, and H₂S concentrations at all sites used in the reciprocal translocation experiments. Measurements were performed within as well as outside of experimental containers. All values represent means \pm standard deviations.

	Temperature [°C]	pH	Specific conductivity [μ S]	Dissolved oxygen [mg/l]	H ₂ S [μ M]
<u>Experiment 1: non-sulphidic vs. sulphidic habitats</u>					
Non-sulphidic habitat (outside of buckets), <i>N</i> =6	23.9 \pm 1.1	8.0 \pm 0.2	342 \pm 26	5.26 \pm 0.89	-0.4 \pm 1.2 ¹
Non-sulphidic habitat (inside of buckets), <i>N</i> =6	24.0 \pm 0.7	7.9 \pm 0.1	337 \pm 31	5.11 \pm 0.58	-0.2 \pm 2.0 ¹
Sulphidic habitat (outside of buckets), <i>N</i> =6	27.9 \pm 0.3	7.2 \pm 0.1	3,987 \pm 41	1.27 \pm 0.09	21.3 \pm 11.2
Sulphidic habitat (inside of buckets), <i>N</i> =6	28.1 \pm 0.1	7.1 \pm 0.0	4,002 \pm 36	1.21 \pm 0.10	17.1 \pm 9.4
<u>Experiment 2: surface vs. cave habitats</u>					
Surface habitat (outside of buckets), <i>N</i> =6	28.0 \pm 0.3	7.0 \pm 0.1	3,972 \pm 39	1.24 \pm 0.07	19.7 \pm 16.4
Surface habitat (inside of buckets), <i>N</i> =6	28.2 \pm 0.2	7.1 \pm 0.1	3,897 \pm 51	1.26 \pm 0.07	18.3 \pm 12.9
Cave habitat (outside of buckets), <i>N</i> =6	28.1 \pm 0.0	7.0 \pm 0.0	4,125 \pm 89	1.38 \pm 0.21	24.7 \pm 9.6
Cave habitat (inside of buckets), <i>N</i> =6	28.1 \pm 0.0	7.0 \pm 0.1	4,188 \pm 62	1.25 \pm 0.11	22.4 \pm 13.0

¹The spectrophotometric measurement of sulphide has an error margin of about \pm 5 μ M. Consequently, these samples likely do not contain any H₂S.

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