

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

M. TOBLER*, R. RIESCH†, C. M. TOBLER‡, T. SCHULZ-MIRBACH§ & M. PLATH¶

*Department of Biology and Department of Wildlife and Fisheries Sciences, Texas A&M University, College Station, TX, USA

†Department of Zoology, University of Oklahoma, Norman, OK, USA

‡Department of Entomology, Texas A&M University, College Station, TX, USA

§Department of Earth and Environmental Sciences, Section Palaeontology, Ludwig-Maximilians-University Munich, Munich, Germany

¶Department of Ecology & Evolution, Goethe-University Frankfurt, Frankfurt a. M., Germany

Keywords:

cave fish;
ecological speciation;
female choice;
hydrogen sulphide;
isolation-by-adaptation;
reciprocal translocation experiment;
reproductive isolation.

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).

Correspondence: Michael Tobler, Department of Wildlife and Fisheries Sciences, Texas A&M University, 2258 TAMU, College Station, TX 77843, USA.
Tel.: +1 979 847 8846; fax: +1 979 845 5391; e-mail: michi.tobler@gmail.com

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.

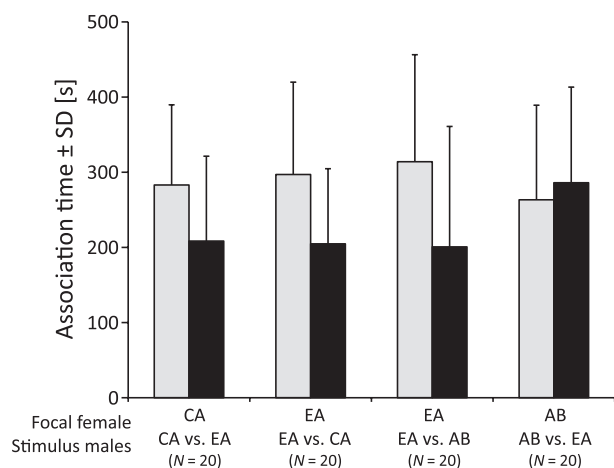


Fig. 1 Mean (\pm SD) association times of females with a male of the same population (light grey bars) and a male from an adjacent but ecologically divergent habitat (dark grey bars). CA: Cueva del Azufre (sulphidic cave); EA: El Azufre (sulphidic surface habitat); AB: Arroyo Bonita (nonsulphidic surface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

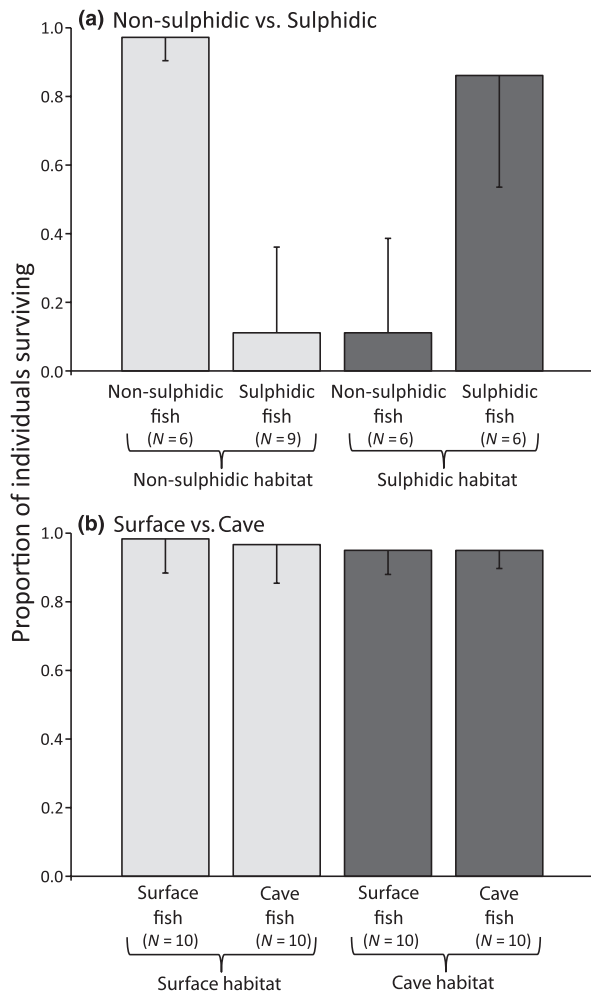


Fig. 2 Mean (\pm SD) survival of *Poecilia mexicana* in translocation experiments between nonsulphidic and sulphidic surface habitats (a) and sulphidic cave and surface habitats (b). Each replicate consisted of six individual fish that were kept in 20-L confinements.

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.

Acknowledgments

The experiments reported in this paper comply with the current legislation of the European Union and the US. The Mexican government kindly issued permits (DGOPA.06192.240608-1562). We thank A. Bötger, N. Bunzel, V. Duwe, N. Herrmann, J. Horstkotte, C. Kaufman, L. Padur, A. Pease, A. Ramm, K. Scharnweber, M. Schulte and M. Ziege for assistance in the field. We are also indebted to G. Rosenthal, I. Schupp and K. Winemiller for their continuous support. Funding came from the Swiss National Science Foundation

(PBZHA-121016), the German Research Foundation (PL470/1-2) and the US National Science Foundation (#105095200).

References

- Bagarinao, T. 1992. Sulfide as an environmental factor and toxicant: tolerance and adaptations in aquatic organisms. *Aquat. Toxicol.* **24**: 21–62.
- Chen, K. & Morris, J. 1972. Kinetics of oxidation of aqueous sulfide by O₂. *Environ. Sci. Technol.* **6**: 529–537.
- Cooper, R.U., Clough, L.M., Farwell, M.A. & West, T.L. 2002. Hypoxia-induced metabolic and antioxidant enzymatic activities in the estuarine fish *Leiostomus xanthurus*. *J. Exp. Mar. Biol. Ecol.* **279**: 1–20.
- Curtis, C., Bartholomew, T., Rose, F. & Dodgson, K. 1972. Detoxication of sodium ³⁵S-sulphide in the rat. *Biochem. Pharmacol.* **21**: 2313–2321.
- Dettman, J., Sirjusingh, C., Kohn, L. & Anderson, J. 2007. Incipient speciation by divergent adaptation and antagonistic epistasis in yeast. *Nature* **447**: 585–588.
- Funk, D.J. 1998. Isolating a role for natural selection in speciation: host adaptation and sexual isolation in *Neochlamisus bebbianae* leaf beetles. *Evolution* **52**: 1744–1759.
- Gordon, M.S. & Rosen, D.E. 1962. A cavernicolous form of the poeciliid fish *Poecilia sphenops* from Tabasco, México. *Copeia* **1962**: 360–368.
- Grant, P.R. & Grant, B.R. 2008. Pedigrees, assortative mating and speciation in Darwin's finches. *Proc. R. Soc. Lond. B Biol. Sci.* **275**: 661–668.
- Grieshaber, M.K. & Völkel, S. 1998. Animal adaptations for tolerance and exploitation of poisonous sulfide. *Annu. Rev. Physiol.* **60**: 33–53.
- Halliwell, B. & Gutteridge, M. 1999. *Free Radicals in Biology and Medicine*, 3rd edn. Oxford Science Publication, Oxford, NY.
- Hendry, A.P. 2004. Selection against migrants contributes to the rapid evolution of ecologically dependent reproductive isolation. *Evol. Ecol. Res.* **6**: 1219–1236.
- Hermes-Lima, M. & Zenteno-Savin, T. 2002. Animal response to drastic changes in oxygen availability and physiological oxidative stress. *Comp. Biochem. Physiol. C* **133**: 537–556.
- Houde, A.E. 1997. *Sex, Color, and Mate Choice in Guppies*. Princeton University Press, Princeton, NJ.
- Kawecki, T.J. & Ebert, D. 2004. Conceptual issues in local adaptation. *Ecol. Lett.* **7**: 1225–1241.
- Kimura, H. 2002. Hydrogen sulfide as a neuromodulator. *Mol. Neurobiol.* **26**: 13–19.
- Langerhans, R.B., Gifford, M. & Joseph, E. 2007. Ecological speciation in *Gambusia* fishes. *Evolution* **61**: 2056–2074.
- Lorch, P.D., Proloux, S., Rowe, L. & Day, T. 2003. Condition dependent sexual selection accelerates adaptation by natural selection. *Evol. Ecol. Res.* **5**: 867–881.
- Ludlow, A.M. & Magurran, A.E. 2006. Gametic isolation in guppies (*Poecilia reticulata*). *Proc. R. Soc. Lond. B Biol. Sci.* **273**: 2477–2482.
- Marcia, M., Ermler, U., Peng, G. & Michel, H. 2009. The structure of *Aquifex aeolicus* sulfide:quinone oxidoreductase, a basis to understand sulfide detoxification and respiration. *Proc. Natl Acad. Sci. USA* **106**: 9625–9630.
- Matute, D.R., Novak, C.J. & Coyne, J. 2009. Temperature-based extrinsic reproductive isolation in two species of *Drosophila*. *Evolution* **63**: 595–612.
- Nordlie, F.G. 2006. Physicochemical environments and tolerances of cyprinodontoid fishes found in estuaries and salt marshes of eastern North America. *Rev. Fish Biol. Fish.* **16**: 51–106.
- Nosil, P., Crespi, B. & Sandoval, C. 2002. Host-plant adaptation drives the parallel evolution of reproductive isolation. *Nature* **417**: 440–443.
- Nosil, P., Vines, T.H. & Funk, D.J. 2005. Reproductive isolation caused by natural selection against immigrants from divergent habitats. *Evolution* **59**: 705–719.
- Nosil, P., Funk, D.J. & Ortiz-Barrientos, D. 2009a. Divergent selection and heterogeneous genomic divergence. *Mol. Ecol.* **18**: 375–402.
- Nosil, P., Harmon, L.J. & Seehausen, O. 2009b. Ecological explanations for (incomplete) speciation. *Trends Ecol. Evol.* **24**: 145–156.
- Olsvik, P.A., Kristensen, T., Waagbo, R., Tollefsen, K.E., Rosse-land, B.O. & Toften, H. 2006. Effects of hypo- and hyperoxia on transcription levels of five stress genes and the glutathione system in liver of Atlantic cod *Gadus morhua*. *J. Exp. Biol.* **209**: 2893–2901.
- Parzefall, J. 1970. Morphologische Untersuchungen an einer Höhlenform von *Mollienesia sphenops* (Pisces, Poeciliidae). *Z. Morph. Tiere* **68**: 323–342.
- Parzefall, J. 1979. Genetics and biological significance of the aggressive behavior of *Poecilia sphenops* (Pisces, Poeciliidae): studies on hybrids of epigeous and hypogeous living populations. *Z. Tierpsychol.* **50**: 399–422.
- Parzefall, J. 2001. A review of morphological and behavioural changes in the cave molly, *Poecilia mexicana*, from Tabasco, Mexico. *Environ. Biol. Fish.* **62**: 263–275.
- Peters, N., Peters, G., Parzefall, J. & Wilkens, H. 1973. Über degenerative und konstruktive Merkmale bei einer phylogenetisch jungen Höhlenform von *Poecilia sphenops* (Pisces, Poeciliidae). *Int. Rev. Ges. Hydrobiol.* **58**: 417–436.
- Plath, M. 2008. Male mating behavior and costs of sexual harassment for females in cavernicolous and extremophile populations of Atlantic mollies (*Poecilia mexicana*). *Behaviour* **145**: 73–98.
- Plath, M. & Tobler, M. in press. The evolutionary ecology of the cave molly (*Poecilia mexicana*) from the Cueva del Azufre system. In: *The Biology of Subterranean Fishes* (E. Trajano, M.E. Bichuette & B.G. Kapoor, eds), Science Publishers, Enfield, NH.
- Plath, M., Parzefall, J., Körner, K. & Schlupp, I. 2004. Sexual selection in darkness? Female mating preferences in surface- and cave-dwelling Atlantic mollies, *Poecilia mexicana* (Poeciliidae, Teleostei). *Behav. Ecol. Sociobiol.* **55**: 596–601.
- Plath, M., Hauswaldt, S., Moll, K., Tobler, M., Garcia de Leon, F., Schlupp, I. & Tiedemann, R. 2007a. Local adaptation and pronounced genetic differentiation in an extremophile fish, *Poecilia mexicana*, inhabiting a Mexican cave with toxic hydrogen sulfide. *Mol. Ecol.* **16**: 967–976.
- Plath, M., Tobler, M., Riesch, R., Garcia de Leon, F.J., Giere, O. & Schlupp, I. 2007b. Survival in an extreme habitat: the role of behaviour and energy limitation. *Naturwissenschaften* **94**: 991–996.
- Riesch, R., Tobler, M., Plath, M. & Schlupp, I. 2009. Offspring number in a livebearing fish (*Poecilia mexicana*, Poeciliidae): reduced fecundity and reduced plasticity in a population of cave mollies. *Environ. Biol. Fish.* **84**: 89–94.

- Rodriguez, R., Sullivan, L. & Cocroft, R. 2004. Vibrational communication and reproductive isolation in the *Enchenopa binotata* species complex of treehoppers (Hemiptera: Membracidae). *Evolution* **58**: 571–578.
- Rundle, H.D. & Nosil, P. 2005. Ecological speciation. *Ecol. Lett.* **8**: 336–352.
- Rundle, H.D., Nagel, L., Boughman, J. & Schluter, D. 2000. Natural selection and parallel speciation in sympatric sticklebacks. *Science* **287**: 306–308.
- Ryan, M.J., Dries, L.A., Batra, P. & Hillis, D.M. 1996. Male mate preferences in a gynogenetic species complex of Amazon mollies. *Anim. Behav.* **52**: 1225–1236.
- Schlupp, I., Parzefall, J. & Schartl, M. 2002. Biogeography of the Amazon molly, *Poecilia formosa*. *J. Biogeogr.* **29**: 1–6.
- Schluter, D. 2000. *The Ecology of Adaptive Radiation*. Oxford University Press, Oxford.
- Schluter, D. 2001. Ecology and the origin of species. *Trends Ecol. Evol.* **16**: 372–380.
- Shahak, Y. & Hauska, G. 2008. Sulfide oxidation from cyanobacteria to humans: sulfide-quinone oxidoreductase (SQR). In: *Advances in Photosynthesis and Respiration*, Vol. 27 (R. Hell, C. Dahl, D.B. Knaff & T.L. Leustek, eds), pp. 319–335. Springer, Heidelberg.
- Sies, H. 1986. Biochemistry of oxidative stress. *Angew. Chem. Int. Ed. Engl.* **25**: 1058–1071.
- Snowberg, L.K. & Benkman, C.W. 2007. The role of marker traits in the assortative mating within red crossbills, *Loxia curvirostra* complex. *J. Evol. Biol.* **20**: 1924–1932.
- Snowberg, L.K. & Benkman, C.W. 2009. Mate choice based on a key ecological performance trait. *J. Evol. Biol.* **22**: 762–769.
- Svensson, E., Eroukhmanoff, F. & Friberg, M. 2006. Effects of natural and sexual selection on adaptive population divergence and premating isolation in a damselfly. *Evolution* **60**: 1242–1253.
- Tobler, M. 2008. Divergence in trophic ecology characterizes colonization of extreme habitats. *Biol. J. Linn. Soc.* **95**: 517–528.
- Tobler, M. 2009. Does a predatory insect contribute to the divergence between cave- and surface adapted fish populations? *Biol. Lett.* **5**: 506–509.
- Tobler, M., Schlupp, I., Heubel, K., Riesch, R., Garcia de Leon, F.J., Giere, O. & Plath, M. 2006. Life on the edge: hydrogen sulfide and the fish communities of a Mexican cave and surrounding waters. *Extremophiles* **10**: 577–585.
- Tobler, M., DeWitt, T.J., Schlupp, I., Garcia de Leon, F.J., Herrmann, R., Feulner, P., Tiedemann, R. & Plath, M. 2008a. Toxic hydrogen sulfide and dark caves: phenotypic and genetic divergence across two abiotic environmental gradients in *Poecilia mexicana*. *Evolution* **62**: 2643–2649.
- Tobler, M., Schlupp, I. & Plath, M. 2008b. Does divergence in female mate choice affect male size distribution in two cave fish populations? *Biol. Lett.* **4**: 452–454.
- Verzijden, M. & ten Cate, C. 2007. Early learning influences species assortative mating preferences in Lake Victoria cichlid fish. *Biol. Lett.* **3**: 134–136.
- Walters, L. & Walters, V. 1965. Laboratory observations on a cavernicolous poeciliid from Tabasco, Mexico. *Copeia* **1965**: 214–233.
- Wong, B.B.M., Fisher, H.S. & Rosenthal, G.G. 2005. Species recognition by male swordtails via chemical cues. *Behav. Ecol.* **16**: 818–822.
- Zenteno-Savin, T., Saldierna, R. & Ahuejote-Sandoval, M. 2006. Superoxide radical production in response to environmental hypoxia in cultured shrimp. *Comp. Biochem. Physiol. C* **142**: 301–308.

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.

As a service to our authors and readers, this journal provides supporting information supplied by the authors. Such materials are peer-reviewed and may be re-organized for online delivery, but are not copy-edited or typeset. Technical support issues arising from supporting information (other than missing files) should be addressed to the authors.

Received 19 June 2009; revised 4 August 2009; accepted 2 September 2009