Translocation of cave fish (*Poecilia mexicana*) within and between natural habitats along a toxicity gradient

Ingo Schlupp¹, Timothy J. Colston², Brandon L. Joachim¹, Rüdiger Riesch³

¹Department of Biology, University of Oklahoma, 730 Van Vleet Oval, Norman, OK 73019, USA
²Biology Department, University of Mississippi, Box 1848, University, MS 38677, USA
³Department of Biology, North Carolina State University, 127 David Clark Labs, Raleigh, NC 27695-7617, USA

Abstract – During ecological speciation, diverging taxa have the potential to remain in close spatial proximity (i.e., sympatry or micro-allopatry) theoretically allowing for continued contact and gene flow. In a system where incipient speciation of populations of the Atlantic molly (*Poecilia mexicana*) appears to be driven by abiotic factors, we investigated whether one of these factors, hydrogen sulphide (H₂S) toxicity, also constitutes an effective barrier to slow migration within and between habitats. We addressed this experimentally by translocating individuals from high toxicity to lower toxicity within a toxic cave and by translocating individuals from the toxic cave to a nontoxic surface habitat. Using a stepwise-backwards Cox regression, we found that overall mortality was low, but statistically significant mortality occurred when individuals were transferred from higher toxicity to lower toxicity. In addition, only males were negatively affected by being transferred from low levels of toxicity to nontoxic, surface waters. This indicates that in addition to abiotic factors, other mechanisms, like predation and sexual selection, must be important in maintaining population separation.

Key words: ecological speciation; divergence; habituation; acclimation; troglobite

Introduction

While it has long been accepted that geographic isolation can lead to speciation (i.e., allopatric speciation; Mayr 1963; Coyne & Orr 2004), a number of mechanisms, including sexual (Maan & Seehausen 2011) and natural selection (Schluter 1993; Riesch et al. 2011b), can lead to population divergence and eventually to ecological speciation even in sympatry (Rundle & Nosil 2005). Once populations show signs of phenotypic and genetic divergence, and are on an evolutionary trajectory that could lead to speciation, mechanisms must be in place to reduce the probability of reticulation, or the collapse of the nascent species (Woehrer et al. 2012). Mechanisms responsible for maintaining species separation were potentially also important in generating divergence (but see Coyne & Orr 2004), and thus studying these mechanisms in detail likely also advances our understanding of how sympatric speciation occurs.

Abiotic factors often play an important role in maintaining population separation and divergence (Tobler et al. 2006). However, these factors often represent soft gradients, rather than sharp clines (i.e., abrupt demarcation with presence of a factor on one side and absolute absence of that factor on the other), along which populations are positioned. It is important to understand to which degree these different gradients contribute to divergence, and a sharper cline is thought to lead to conditions under which divergence can evolve easier (Nosil et al. 2009a,b). Classical work in botany, for example, has established how important sharp clines in soil composition can be in population divergence (Dickinson & Antonovics 1973; Antonovics & Thrall 1994). A combination of sharp and soft gradients, on the other
hand, along which organisms can potentially move up and down, occur in a cluster of populations of the Atlantic molly (*Poecilia mexicana*) from Tabasco, Mexico (Tobler & Plath 2011). Here, a population of Atlantic mollies has colonised a toxic cave, the Cueva del Azufre (or Cueva de Villa Luz), that has been under study since its scientific discovery in the 1960s (Gordon & Rosen 1962; Parzefall 2001). The Cueva del Azufre is divided into 13 different chambers, with chamber XIII being the innermost chamber (Gordon & Rosen 1962). Several springs in the cave (mainly in chamber X) release water that is extremely rich in hydrogen sulphide, thus forming a toxicity gradient within the cave that varies in levels of hydrogen sulphide depending on the relative position to the springs (Tobler et al. 2006). As the toxic water flows downstream in the cave, it is oxygenated and, as a result, becomes less toxic with greater distance from the springs. The creek that flows through the cave eventually leaves the cave and becomes the toxic El Azufre, a surface creek also containing hydrogen sulphide. While toxicity represents a somewhat soft gradient, the change from darkness within the cave to light at the mouth of the cave is abrupt. The community of organisms inhabiting the waters of the Cueva del Azufre and El Azufre is extremely depauperate given its geographic location in the tropics (Gordon & Rosen 1962). The Atlantic molly is the only fish species that has permanently colonised the spring habitats within the cave (Tobler et al. 2006; Riesch et al. 2009). Populations of *P. mexicana* inhabiting the toxic cave are fundamentally different from surface-living populations in almost every aspect of their natural history (e.g., behaviour: Plath et al. 2004; morphology: Tobler et al. 2008a; life history: Riesch et al. 2010b), and using common-garden experiments, these differences have repeatedly been shown to have a strong heritable component (e.g., behaviour: Parzefall 2001; morphology: Tobler et al. 2008a; life history: Riesch et al. 2011a; Physiology: Plath & Tobler 2010). Thus, the combined evidence indicates a system in which ongoing, incipient speciation is occurring (Plath et al. 2007a; Tobler & Plath 2011). Clearly such early splitting events are labile and vulnerable to collapse if conditions change (Wu & Ting 2004; Hendry 2009; Nosil et al. 2009a,b; Bolnick 2011; Yeaman & Whitlock 2011; Woehrer et al. 2012), although so far we have no evidence for this happening in *P. mexicana*. In fact, we have strong evidence indicating that population divergence is relatively stable because phenotypic and genetic divergence has remained strong even if faced by strong temporary disturbance due to natural catastrophes (like extreme flooding events; Plath et al. 2010). This said, there are other outcomes possible, including stable divergence with limited gene flow and selection, which would enable us to study the underlying genomic architecture of divergence (Nosil 2012).

Previous work on mechanisms isolating the cave and the surface populations has documented that sudden translocation of surface fish into nonfamiliar habitat (i.e., toxic to nontoxic and vice versa) leads to high mortality (Tobler et al. 2009; Plath et al. 2010), while sudden translocation within toxic habitats (i.e., toxic surface to toxic cave and vice versa) yielded no measurable mortality (Tobler et al. 2009). Thus, toxicity limits successful migration and promotes isolation between fish from nontoxic and toxic habitats. The proposed mechanism behind the high mortality is physiological incompatibility with the new conditions for either form (Tobler et al. 2009). Nonetheless, this result comes as somewhat of a surprise, because over the last couple of decades, experimental populations of *P. mexicana* have successfully and repeatedly been established from toxic (surface and cave) habitats in benign (i.e., nontoxic) laboratory environments after acclimation to nontoxic water. Why then was mortality in the field experiments so high? This could be due to the sudden exposure to either relatively high levels of hydrogen sulphide (for fish from nontoxic waters) or relatively high levels of oxygen (for fish from toxic waters) following translocation (Tobler et al. 2009).

Thus, while previous translocation experiments have addressed how well individuals would fair after rapid transport into a foreign environment, the more plausible scenario of slow and gradual acclimation of individuals to a new habitat has so far not been considered experimentally. As toxicity in the cave molly system represents a gradient, one can hypothesise that slower, more gradual movement along the gradient would allow individuals a higher probability of survival when migrating from more toxic to less toxic conditions. This question is also important relative to the surprisingly strong genetic structuring within the toxic cave. While there is strong genetic structure even between chambers within the Cueva del Azufre, there is, nonetheless, evidence for low levels of gene flow between different cave chambers. On the other hand, we have so far found no evidence for any gene flow from migrants between the toxic surface and toxic cave on one side and the nontoxic surface habitats on the other side, further corroborated by *F* _ST_ values that are usually above 0.2 for any possible pairwise comparison (Plath et al. 2007a, 2010; Tobler et al. 2008a, 2009). For example, Tobler et al. 2009 were able to demonstrate that, based on microsatellite DNA, less than 10% first-generation migrants move from the inside of the Cueva del Azufre into the adjacent toxic surface stream, while first-generation migrants within
these habitats (e.g., between different cave chambers) ranged anywhere from 5 to 35%. Interestingly, a study investigating the population genetic structure, following a millennium flood in 2007 (Plath et al. 2010), found slightly higher numbers of migrants within the cave immediately after the event, pointing to a potential role of rare but catastrophic events. What maintains population structuring between (and within) the Cueva del Azufre and the nontoxic surface habitats (Plath et al. 2007a, 2010; Tobler et al. 2008a)? Are individuals so well adapted to the local conditions that dispersal even within the cave is limited?

In this study, we addressed the following questions: how do fish fair if they are translocated along the toxicity gradient within the cave, where the other environmental gradient (darkness) remains unchanged? Furthermore, does acclimation to the local conditions play a role when fish are transferred from the toxic cave to nontoxic surface waters? Finally, do the sexes respond differently to translocations? To tackle these questions, we conducted three separate translocation experiments, all of which were designed to take individuals from higher toxicity to lower toxicity. In the first experiment, we translocated fish from the very toxic chamber X to the less toxic chamber V. In our second experiment, we moved individuals from chamber V to the even less toxic chamber II, and finally, in a third experiment, we translocated fishes from chamber V to a nontoxic surface habitat after acclimating them to nontoxic water overnight.

Materials and methods

Study system

All experiments were conducted in January 2010 near Tapijulapa, Tabasco, Mexico. We selected fish from habitats containing high hydrogen sulphide concentrations (chamber X: 91.5 ± 32.3 μM), low concentrations of hydrogen sulphide (chamber V: 28.4 ± 15.3 μM) and very low concentrations of hydrogen sulphide (chamber II: 24.0 ± 21.6 μM) (Tobler et al. 2006). Additionally, fish were translocated to a nontoxic surface stream, the Arroyo Bonita (17°25′37.42″N, 92°45′6.98″W). The Arroyo Bonita is approximately 2 km upstream from where the outflow of the Cueva del Azufre connects to the Río Oxolotán, and does not contain hydrogen sulphide (Tobler et al. 2008a).

Local adaptation and natural selection

Fish were collected by hand with small dip nets in each cave chamber to minimise stress from handling and because seining is impossible due to the topography of the cave. Fishes were subsequently moved to individual containers (30.48 × 8.89 × 17.78 cm) (L × H × W) and placed in cave chambers that featured lower concentrations of H₂S than the local area of collection (i.e., chamber X fish were moved to chamber V; chamber V fish were moved to chamber II and chamber V fish moved to the surface habitat). As a control, fishes from chamber V were captured and placed in individual containers within the same chamber. We aimed for an even sex ratio in the experimental fish, but were somewhat limited by the fish available in the respective chambers (Table 1).

For the experiment in which chamber V fish were translocated to the surface habitat, we used a modified protocol. Fishes were collected as described, kept in two 19-litre coolers in their habitat-specific water. This water was aerated for 4 h using portable air pumps, which slowly removed hydrogen sulphide and oxygenized the water. After 4 h, we gradually replaced the cave water with river water from the nearby Río Oxolotán, a freshwater surface habitat. The next morning fish were then placed in individual containers in a surface habitat, Arroyo Bonita, a small tributary to the Río Oxolotán (both the Río Oxolotán and the Arroyo Bonita are naturally populated by the surface form of P. mexicana). Overnight mortality was low; during our overnight acclimation period of chamber V fish, only one male died.

Previous studies have utilised large containers (buckets with holes drilled into them) with multiple fish in the same container (Tobler et al. 2009). However, we chose to use smaller individual containers to minimise any stress that may be associated with crowding. Additionally, small holes were drilled in each container to allow for the circulation of water. Each container contained one fish and was weighed down with small rocks that were gathered locally. Containers were placed in a shallow area with low flow to avoid having the containers washed away. Due to the low oxygen concentrations in the Cueva del Azufre, cavernicolous P. mexicana spend the majority of their time at the water’s surface performing aquatic surface respiration (ASR) to exploit increased oxygen levels (Tobler et al. 2006). Therefore, we did not fully submerge our containers to avoid mortality that could be attributed to behavioural limitations. Once all fish were placed in individual containers, we allowed 1 h for acclimatisation and subsequently checked the condition of the fish in each habitat (see Table 1). At the termination of each experiment (after 24 h), fish were removed from containers, sacrificed and preserved in 5% formaldehyde to avoid inadvertent mixing of populations. All individuals used in the experiment were adult. For
logistical reasons, we were unable to conduct a study that would have measured mortality over a longer term. Similarly, we were unable to conduct a control experiment within chamber X.

Statistical analysis

We used a stepwise-backwards Cox regression based on likelihood ratios to analyse individual survival of *P. mexicana* as implemented in IBM® SPSS® Statistics 19.0.0 (IBM Corporation). In the original model, ‘treatment’ (PSX-to-PSV, PSV-to-PSV, PSV-to-PSII and PSV-to-AB), ‘sex’ (male vs. female) and the interaction of ‘treatment-by-sex’ were included as covariates.

Results

When analysing the survival of cave-dwelling *P. mexicana* in different habitats, the covariate ‘sex’ (*P* = 0.868) was removed from the final Cox regression model during the stepwise-backwards approach. Only the covariates ‘treatment’ (B = 0.84, SE = 0.39, Wald = 4.69, d.f. = 1, *P* = 0.03) and the interaction term ‘treatment-by-sex’ (B = 0.58, SE = 0.29, Wald = 3.91, d.f. = 1, *P* = 0.048) were retained as statistically significant in the final model (−2 log likelihood = 127.42).

Overall we detected very low mortality across treatments. No mortality was uncovered in two treatments (PSV-to-PSV and PSV-to-PSII), while some mortality occurred in the other two treatments (PSX-to-PSV and PSV-to-AB, Fig. 1; significant ‘treatment’ effect). Furthermore, while males and females suffered roughly similar mortality rates when transferred from highly toxic waters (PSX) to low levels of toxicity (PSV), only males were negatively affected by being transferred from low levels of toxicity (PSV) to nontoxic waters (AB, Fig. 1; significant ‘treatment-by-sex’ effect).

Discussion

How animals can move along relevant environmental gradients is important for our understanding of divergence and ultimately ecological speciation. Sharp clines, like the difference between dark and light in the cave molly system, are much more likely to aid population separation than soft gradients (Nosil et al. 2009a,b), as exemplified by the hydrogen sulphide gradient known for the cave molly within the Cueva del Azufre. In our particular system, we examined how differences in hydrogen sulphide toxicity affect the survival of immigrants, and therefore population structuring, between populations within the Cueva del Azufre. Previous research indicated that abrupt changes in water conditions (i.e., toxic to nontoxic and vice versa) lead to very high mortality in translocated fishes, but that translocation between chamber II and the El Azufre (i.e., toxic cave and toxic surface) resulted in no measurable mortality (Tobler et al. 2009). In the experiment presented here, in which we tested for survivorship during gradual migration from highly toxic to lesser toxic microhabitats (i.e., cave chambers) within the cave, we found low levels of mortality in two of four treatments. This indicates that differences in toxicity found within the
cave do not fully explain the genetic structure uncovered by previous studies (Plath et al. 2007a, 2010; Tobler et al. 2008a). Clearly, factors other than the toxicity gradient must play a role here in limiting between-chamber movement, and we can only speculate that they could include sexual selection, predation and age-specific mortality. Furthermore, we found that translocation from a toxic cave habitat to a nontoxic surface habitat results in relatively low mortality if the water is slowly detoxified and fish have time to adjust to the new conditions.

Interestingly, males turned out to be significantly more likely to die than females, when translocated from toxic waters within the cave to a nontoxic surface water habitat. Sex-specific responses to physiological stress are widespread among animal taxa (e.g., insects: Freitak et al. 2012; rats: Sterrenburg et al. 2011; humans: Verma et al. 2011), and even though this depends on the taxa under investigation and the stressors involved, males often seem to be more sensitive to stressful conditions (Freitak et al. 2012; but see Riesch et al. 2011b). In our system, it is not clear what exactly is causing this. Nonetheless, our finding is congruent with data from previous field experiments in this system in which males usually also performed worse than females (Plath et al. 2007a,b). Likely males are physiologically less capable of buffering adverse conditions than females, potentially due to the lower body condition of cave molly males exhibited within the Cueva del Azufre, indicating the males might be more energy limited than females (Riesch et al. 2010b, 2011a,b). Additional indirect evidence for this comes from studies investigating male–male aggression and the propensity of males to engage in sexual harassment of females (e.g., Parzefall 2001; Bierbach et al. 2012). While these behaviours are widespread in *P. mexicana* from benign habitats, such costly behaviours are highly reduced in the Cueva del Azufre, likely as an adaptation to preserve much needed energy for reproduction (Parzefall 2001; Bierbach et al. 2012). Finally, while our results could also indicate that buffering ability might potentially increase with size (and *P. mexicana* males are on average smaller than females; Riesch et al. 2010b, 2011a,b), this interpretation is unlikely because a previous study has shown that smaller adults actually have a higher sulphide tolerance than larger individuals (Tobler et al. 2011b).

In summary, our experiments indicate that under certain conditions (i.e., gradual transfer along the gradient from toxic to nontoxic), factors other than toxicity must also play an important role in maintaining reproductive isolation reported on by previous studies (Plath et al. 2007a, 2010; Tobler et al. 2008a). Clearly, sudden translocation as it is possible during extreme flooding events (Plath et al. 2010) or human-induced translocation during a religious ceremony, in which cave fishes are poisoned and immobilized resulting in passive downstream drifting (Tobler et al. 2011a), is not likely to lead to successful gene flow due to the high mortality induced by rapid translocation between extreme and benign environments (Tobler et al. 2009). Under the scenario that we investigated here that envisions gradual movement along a gradient allowing for potential acclimatisation, previously described additional selective forces acting against migrants (e.g., predation: Tobler 2009; Riesch et al. 2010a; sexual selection: Tobler et al. 2008b) are likely to contribute more to reproductive isolation than previously assumed (Tobler 2009). Finally, the higher survival of females, nonetheless, raises the interesting possibility of sex-specific dispersal that should be investigated further in future studies.

Acknowledgements

We are very grateful to the people and authorities of Tapijula and the Federal authorities of Mexico for granting permits. Francisco García de León and Lenin Arias-Rodriguez helped logistically and with fieldwork. Funding for this project came from NSF (DEB-0743406). This work was conducted under University of Oklahoma IACUC R09-030. We are also thankful to the reviewers who helped greatly to improve the manuscript. IS gratefully acknowledges support from the Alexander von Humboldt Foundation.

References


