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An indigenous religious ritual selects for resistance to a toxicant in a livebearing fish

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Human-induced environmental change can affect the evolutionary trajectory of populations. In Mexico, indigenous Zoque people annually introduce barbasco, a fish toxicant, into the Cueva del Azufre to harvest fish during a religious ceremony. Here, we investigated tolerance to barbasco in fish from sites exposed and unexposed to the ritual. We found that barbasco tolerance increases with body size and differs between the sexes. Furthermore, fish from sites exposed to the ceremony had a significantly higher tolerance. Consequently, the annual ceremony may not only affect population structure and gene flow among habitat types, but the increased tolerance in exposed fish may indicate adaptation to human cultural practices in a natural population on a very small spatial scale.

Keywords: adaptation; anthropogenic disturbance; barbasco; cavefish; rotenone; *Poecilia mexicana*

1. INTRODUCTION

Human-induced environmental change affects ecosystems and biodiversity, with extinction being an inevitable consequence for species unable to cope with the altered environmental conditions [1,2]. However, the remarkable ability of organisms to adapt to life in extreme habitats, from permanent ice of the poles to thermal vents in the deep sea [3], is reflected in diverse organisms that rapidly adapt to novel selective pressures in anthropogenically altered environments [4]. Classic examples of evolution in response to human-induced environmental change include industrial melanism [5], grasses adapting to heavy metal pollution [6] and shifts in plant–insect interactions following changes in land-use practices [7]. Human-induced evolution, however, is generally assumed to be unlikely in endangered species with small populations [8], and there are few studies addressing the potential evolutionary consequences of long-term human activities on species with narrow habitat requirements, small distributions and small population sizes. Here, we studied the potential evolutionary effects that a toxicant introduced by an indigenous religious ritual has on fish endemic to caves and sulphide springs.

In the Mexican Cueva del Azufre system, the livebearing fish *Poecilia mexicana* has colonized several habitats that are characterized by the presence or absence of light and toxic hydrogen sulphide. Divergent environmental conditions are driving adaptive divergence of genetically and phenotypically distinct ecotypes, differing in behavioural, life history, morphological and physiological traits [9–11]. Population genetic differentiation is high between habitat types despite close proximity and lack of physical barriers [11,12], resulting in discrete populations in each habitat type. The different ecotypes are highly endemic with small spatial distributions and population sizes; hence, they possess attributes shared by many endangered species.

While natural selection—stemming from a combination of abiotic and biotic environmental factors—undoubtedly played an important role in shaping the different ecotypes, the role of human-induced evolution has not yet been assessed in this system. One of the caves harbouring the unique fish populations is a ceremonial site for the Zoque indigenous people. This opens the possibility to detect evolutionary responses to human cultural practices in natural populations. For centuries, the Zoque have conducted a fertility ceremony asking for rain each year at the end of the dry season during the holy week before Easter, in which they use barbasco plant roots (*Lonchocarpus* sp., Fabaceae) to poison the fish. The anaesthetized fish, believed by the Zoque to be a gift from their gods inhabiting the underworld, are then collected (figure 1*a*) and used as a protein supplement until crops are ready for harvest [13]. Barbasco roots contain the fish poison rotenone, which is a mitochondrial-complex-I inhibitor depressing cellular respiration [14]. Here, we tested whether fish from populations annually exposed to rotenone exhibited a higher resistance to the respiratory toxicant.

2. MATERIAL AND METHODS

This study was conducted in March 2010 before the annual ceremony. During the ceremony, barbasco is deposited inside the cave about 100 m from the cave entrance, from where it is distributed downstream and outside of the cave by the water currents. We collected fish from two sites that are affected by barbasco, and two sites that were unaffected and located upstream of barbasco introduction (figure 1*b*). Both affected and unaffected sites included sulphidic cave ecotypes (sites 2 and 3, which correspond to cave chambers V and X according to Gordon & Rosen [15]) and sulphidic surface ecotypes (sites 1 and 4, which correspond to El Azufre I and II according to Tobler *et al.* [11]), hence naturally occurring H_2S was present at all sites [16]. Even though gene flow is present among some sites, there is significant genetic differentiation, and populations within barbasco groups are not more closely related to one another than populations of different barbasco regimes (electronic supplementary material). Consequently, populations were treated as independent for statistical analyses.

After collection, fish were kept in insulated coolers with water from the collection sites. To standardize the experimental conditions, the water from the collection sites was gradually replaced with aerated well water that did not contain H_2S over the first 12 h after capture, and fish were allowed to acclimatize to these conditions for another 48 h. During this time, the water was continuously aerated and filtered, and fish were fed once a day with commercially available fish food.

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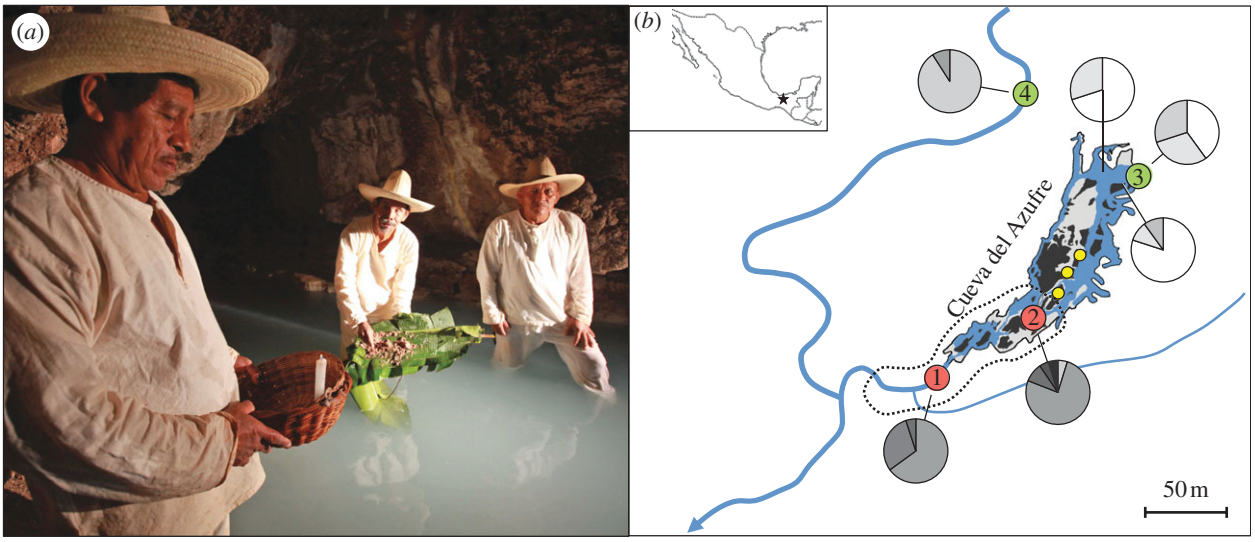

Figure 1. (*a*) Zoque villagers introducing barbasco (here positioned on a leaf) during a religious ceremony. The baskets are used to scoop anaesthetized fish from the water (Photo: Mona Lisa Productions). (*b*) Map of the study area where blue lines represent surface waters, blue areas represent water within the cave (Cueva del Azufre), light grey areas are dry land within the cave and dark areas indicate impenetrable bedrock. The yellow dots highlight the sites of barbasco deposition, and fish located downstream from this site are directly affected by the ceremony (dashed line). For the tolerance experiment, fish were collected from four sites, two of which were within the affected zone (red dots) and two in unaffected areas (green dots). Pie charts depict cytochrome *b* haplotype frequencies across populations (data from Tobler *et al.* [11]).

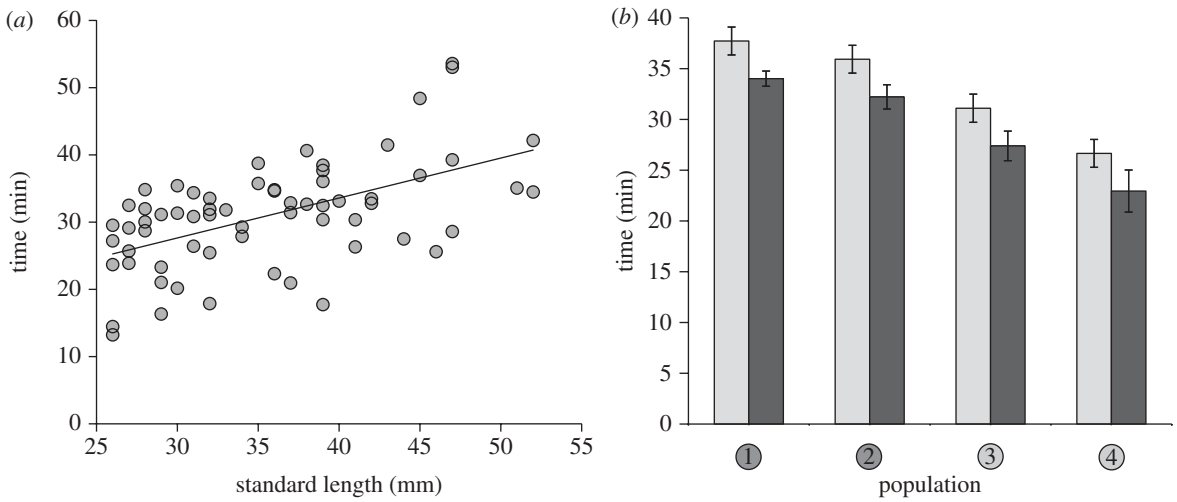

Figure 2. (*a*) Relationship between standard length and time until loss of motion control upon barbasco exposure. Note that the barbasco concentration increased logarithmically as time progressed (electronic supplementary material). (*b*) Estimated marginal means ($\pm$s.e.) for time until loss of motion control in males and females from each population. *Post hoc* tests (Fisher's LSD) indicated that populations 1 and 2 have a significantly higher tolerance than 3 and 4, and 3 has a significantly higher tolerance than 4. (*b*) Grey bars, females; black bars, males.

To compare the tolerance to barbasco between fish from different sites, we obtained barbasco roots from the same stock used for the ceremony. Roots were cut into small pieces (approx. 5 mm cubes), and a stock solution was prepared by soaking 20 g of barbasco in 1000 ml of water for 12 h. For the experiment, individual fish were placed in containers with 100 ml water from the stock tanks, and 5 ml of barbasco stock solution was added at 2 min intervals. This resulted in a logarithmic increase in concentration to 12 mg ml^{-1} over 60 min (electronic supplementary material, figure S1). We measured the time until fish lost motion control. Then fish were removed from the container, measured for standard length, weighed and placed in a heavily aerated recovery container. All fish survived the tolerance test and were eventually released at the original collection site. Data were analysed using ANCOVA, where 'time until loss of motion control' was used as a dependent variable, 'sex' and 'population' as independent variables and 'standard length' as a covariate. Interaction effects were not significant (electronic supplementary material, table S2); hence only main effects were analysed. Standard length and body weight were correlated with each other ($R^2 = 0.855$, $p < 0.001$), and using weight as a covariate qualitatively yielded identical results. Effect strengths were estimated by use of partial eta squared (η_p^2). All statistical analyses were performed using SPSS 17 (SPSS Inc. 2008).

3. RESULTS

We tested a total of eight individuals per sex and population (overall $N = 64$) and found that the time until fish lost motion control was positively correlated with standard length ($F_{1,58} = 38.191$, $p < 0.001$, $\eta_p^2 = 0.397$; figure 2*a*). There was also a significant difference between the sexes ($F_{1,58} = 8.540$, $p = 0.005$, $\eta_p^2 = 0.128$), and males were significantly more

susceptible than females (figure 2*b*). Most importantly, we found significant variation in susceptibility to the effects of barbasco among sites ($F_{3,58} = 16.693$, $p < 0.001$, $\eta_p^2 = 0.463$). Fish in front chambers of the cave and the cave outflow, which are exposed to barbasco during the ceremony, maintained motion longer than those from populations never exposed to barbasco (figure 2*b*).

4. DISCUSSION

Our findings reveal potential effects of an indigenous cultural practice on three distinct processes: (i) dynamics within affected populations, (ii) gene flow among populations, and (iii) adaptive trait divergence between affected and unaffected populations. The annual ceremony may affect attributes of exposed populations because toxic effects were both sex- and size-specific. This might lead to shifts in sex ratios as well as size distributions of affected populations, but this has yet to be confirmed in natural populations. An analysis of dead fish recovered after the 2007 ceremony indicated that sex ratio in dead fish did not deviate significantly from a reference sample, while the size distribution of dead fish was shifted towards a smaller body size as would be expected based on the tolerance tests (electronic supplementary material).

The annual ceremony probably also affects gene flow between surface and cave populations. Previous population genetic analyses indicated that gene flow between habitat types in the Cueva del Azufre system (i.e. between sulphidic and non-sulphidic as well as between cave and surface habitats) is very low, with the exception of unidirectional gene flow between the sulphidic cave and sulphidic surface habitats [11,12]. This coincides with the flow direction and exposure to barbasco. Hence, we suggest that gene flow between the two habitat types may actually be mediated at least in part by the downstream drift of sedated individuals that might be able to reproduce despite natural selection against immigrants in the system [17].

Finally, the ceremony appears to have driven adaptive trait divergence among populations in this system, as fish from sites annually exposed to the ceremony—independent of ecotype—had higher resistance to barbasco than fish from unaffected sites. Further analyses in the laboratory using common-garden experiments will be required to corroborate that differences in barbasco tolerance are due to adaptive trait divergence and to disentangle potential effects of phenotypic plasticity and heritable differentiation. Overall, this study indicates that trait differentiation in this system may not only be affected by the major differences in abiotic environmental conditions, but also by more fine-scale differences within habitat types. Further evidence for this comes from morphological and heritable gene expression gradients within the sulphidic cave [18,19] as well as population genetic structure within habitat types (figure 1*b*) [11,12,20]. In summary, human cultural practices might not only affect population dynamics, but also drive adaptive divergence on small spatial scales.

For this study, the authors adhered to the Guidelines for the Use of Animals in Research. The experiments reported here are in agreement with the respective laws in Mexico (permit: DGOPA.00093.120110.-0018).

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