

Physiological adaptation along environmental gradients and replicated hybrid zone structure in swordtails (Teleostei: *Xiphophorus*)

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Abstract

Local adaptation is often invoked to explain hybrid zone structure, but empirical evidence of this is generally rare. Hybrid zones between two poeciliid fishes, *Xiphophorus birchmanni* and *X. malinche*, occur in multiple tributaries with independent replication of upstream-to-downstream gradients in morphology and allele frequencies. Ecological niche modelling revealed that temperature is a central predictive factor in the spatial distribution of pure parental species and their hybrids and explains spatial and temporal variation in the frequency of neutral genetic markers in hybrid populations. Among populations of parentals and hybrids, both thermal tolerance and heat-shock protein expression vary strongly, indicating that spatial and temporal structure is likely driven by adaptation to local thermal environments. Therefore, hybrid zone structure is strongly influenced by interspecific differences in physiological mechanisms for coping with the thermal environment.

Introduction

Geographic variation in environmental conditions affects survival and reproduction of organisms and determines their distribution across small and large spatial scales (McArthur *et al.*, 1988; Peterson, 1993; Rainey & Travissano, 1998; Slabbekoorn & Smith, 2002; Novembre & Di Rienzo, 2009). As such, environmental variation is a key driver in the evolution of biodiversity, giving rise to genetic and phenotypic variation within species (Mayr, 1963; Endler, 1977; Kawecki & Ebert, 2004; Shepard & Burbrink, 2011) and also mediating the evolution of reproductive isolation, resulting in closely related species being distributed along environmental gradients (e.g. Schluter, 2000; Doebeli & Dieckmann, 2003; Rundle &

Nosil, 2005; Keller & Seehausen, 2012). Environmental gradients not only provide an initial stimulus for divergence, but may also serve as conduits for secondary contact between related species occurring in distinct environments, thus facilitating hybridization. Consequently, hybrid zones commonly coincide with environmental gradients (Fritsche & Kaltz, 2000; Yanchukov *et al.*, 2006; Kameyama *et al.*, 2008), and two types in particular, tension zones and zones of bounded hybrid superiority, tend to occur along gradients in biotic and abiotic variables (Good *et al.*, 2000; Gay *et al.*, 2008; Ruegg, 2008).

The longstanding belief that hybrid zones are evolutionary sinks that inhibit diversification (Mayr, 1963; Wagner, 1970) has been subtly perpetuated due to a focus on tension zones, in which reduced hybrid fitness is balanced by inward dispersal and continual hybridization of parentals (reviewed in Barton & Hewitt, 1985). However, hybridization can be a source of evolutionary

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novelty and produce transgressive or intermediate phenotypes that outperform parentals under certain environmental conditions (Lewontin & Birch, 1966; Seehausen, 2004; Bell & Travis, 2005; Parnell *et al.*, 2008; Tobler & Carson, 2010). These hybrid zones are often referred to as cases of ‘bounded hybrid superiority’ (Moore, 1977) because hybrids can have greater fitness than either parental species in intervening transitional habitats (i.e. ecotones). These cases in which hybrids outperform parental species remain less well understood, even though hybrid superiority is a requisite for homoploid hybrid speciation (e.g. Salzburger *et al.*, 2002; Schliewen & Klee, 2004; Gompert *et al.*, 2006; Mallet, 2007; Jiggins *et al.*, 2008). Elucidating the mechanisms underlying hybrid superiority involves identifying both the traits mediating adaptation and the selective forces that determine fitness in the respective environment (for review, see Arnold & Martin, 2010). This can be particularly challenging because environmental gradients are notoriously complex due to covariation among multiple abiotic and biotic environmental variables (Grether *et al.*, 2001; Reznick *et al.*, 2001; Tobler & Plath, 2011).

Here, we examine a case in which hybrids are abundant in an area of secondary contact between two swordtail fish species occurring along an elevational gradient. In the Río Pánuco basin, along the eastern slopes of the Sierra Madre Oriental of Mexico, *Xiphophorus birchmanni* is distributed throughout the lowlands and foothills, generally below 400 m above sea level. In contrast, *X. malinche* has a strictly highland distribution and is known from only six sites in headwater streams (Rauchenberger *et al.*, 1990; Gutiérrez-Rodríguez *et al.*, 2008; Culumber *et al.*, 2011). Fertile hybrids of the two species are abundant at intermediate elevations (Rosenthal *et al.*, 2003; Culumber *et al.*, 2011). This high–intermediate–low-elevation distribution of *X. malinche*, hybrids and *X. birchmanni* is observed in a replicated fashion in at least seven separate stream reaches (Culumber *et al.*, 2011), indicating that natural selection along the elevational gradient maintains replicated hybrid zone structure. However, it is as yet unknown what environmental factors stabilize the hybrid zones and what traits mediate local adaptation of parentals and hybrids in their respective environments. Additionally, if this is a case of bounded hybrid superiority maintained by abiotic variables, then we would expect to find certain variables that restrict parentals to their areas of occurrence and for which hybrids outperform parentals in their area of occurrence.

In this study, we used an integrative approach to elucidate the role of abiotic environmental conditions, particularly climatic and hydrographic variables, in stabilizing the replicated hybrid zone structure between *X. birchmanni* and *X. malinche*. Our approach included the following: (i) We used ecological niche modelling (ENM) to test whether the distributions of *X. birchmanni*,

X. malinche and their hybrids could be predicted by abiotic environmental variables and whether they occupy different environmental niches. We further used spatially explicit climatic and hydrographic data to compare environments occupied by both parental species and hybrids in order to identify the specific variables that differ among the three groups. (ii) Using the variables found to differ between the two parental species, we tested whether we could predict spatial and temporal variation in species-specific allele frequencies at a finer scale within only hybrid localities. To do this, we genotyped individuals from multiple hybrid populations using single-nucleotide polymorphism (SNP) markers and related allele frequencies with environmental variables to analyse variation in both space and time. If the factors that emerge in spatial analyses do in fact impose selection related to thermal tolerance, then temporal analyses should demonstrate concomitant changes in allele frequencies. (iii) With temperature emerging as a key predictor variable in various analyses, we examined thermal tolerance to compare physiological trait differentiation among parental and hybrid populations. We used a loss-of-equilibrium assay to quantify both heat and cold tolerance of wild-caught parentals and hybrids during summer and winter. We also tested pure parental fish born and reared in a common garden environment to determine whether between-species variation in tolerance can be attributed to genetic differences or to phenotypic plasticity. (iv) Finally, we quantified and compared gene expression of heat-shock proteins (*hsps*), which act as molecular chaperones to buffer against cell damage in response to thermal stress (Feder & Hoffmann, 1999), in wild-caught fish and in laboratory-acclimated fish exposed to acute thermal stress.

Materials and methods

Ecological niche modelling

To evaluate the abiotic environmental factors that influence the distribution of each parental species and their hybrids and test whether they occupy distinct environments, we conducted GIS-based analyses of each species’ environmental niche using ENM techniques (Peterson, 2001; Kozak *et al.*, 2008; Elith & Leathwick, 2009). We obtained locality point data for each species and their hybrids (Table S1) from our own fieldwork (Culumber *et al.*, 2011; GGR and MT unpublished data) and the published literature (Gutiérrez-Rodríguez *et al.*, 2008). Localities where only pure *X. birchmanni* or pure *X. malinche* were observed were classified as *X. birchmanni* and *X. malinche* sites, respectively. Any locality where hybrids were present was classified as a hybrid site. Next, we assembled a set of coverages for 24 environmental variables from the Worldclim (<http://www.worldclim.org>) and Hydro1k (http://eros.usgs.gov/#/Find_Data/

Products_and_Data_Available/gtopo30/hydro) project databases. Data layers were either downloaded or projected at 30 arc-seconds ($\sim 1 \text{ km}^2$) resolution. The Worldclim data set is composed of 19 bioclimatic variables related to temperature, precipitation and seasonality, whereas the Hydro1k data set is composed of five variables related to hydrography (Hijmans *et al.*, 2005; Kozak *et al.*, 2008). The variables in these data sets have been used successfully in previous studies on freshwater fish species employing ENM (e.g. Domínguez-Domínguez *et al.*, 2006; Chen *et al.*, 2007; McNyset, 2009; Costa & Schlupp, 2010). We clipped data layers to a region that included the north-eastern portion of the state of Hidalgo, and adjacent parts of Veracruz, San Luis Potosí and Querétaro states ($20.5\text{--}21.4^\circ \text{ N}$ and $97.9\text{--}99.1^\circ \text{ W}$; Fig. 1). This region encompasses the Río Pánuco and its tributaries and spans an elevation range from 45 to 2527 m above sea level (Fig. 1). This spatial extent should provide adequate information to train the ENMs while also restricting analyses to a geographic area in

which species could possibly occur (Anderson & Raza, 2010; Barve *et al.*, 2011; Elith *et al.*, 2011).

We constructed ENMs for each species and their hybrids using a maximum entropy method implemented in the program Maxent v.3.3.2 (Phillips *et al.*, 2006; Phillips & Dudik, 2008). Maxent uses environmental variables from localities at which a species has been documented previously (i.e. training data) to build a predictive model of where else the species may occur due to the presence of similar environmental conditions (see Elith *et al.*, 2011 for details). The output of Maxent consists of a threshold-independent measure of the overall performance of the model (area under the receiver operating curve or AUC) and a grid map with each cell having an index of suitability between 0 and 1 (logistic output); higher values indicate higher predicted suitability. An AUC value of 0.5 indicates that the predictive model is no better than random, whereas higher AUC values indicate better predictive ability with a value of 1 indicating perfect prediction. Results also include an

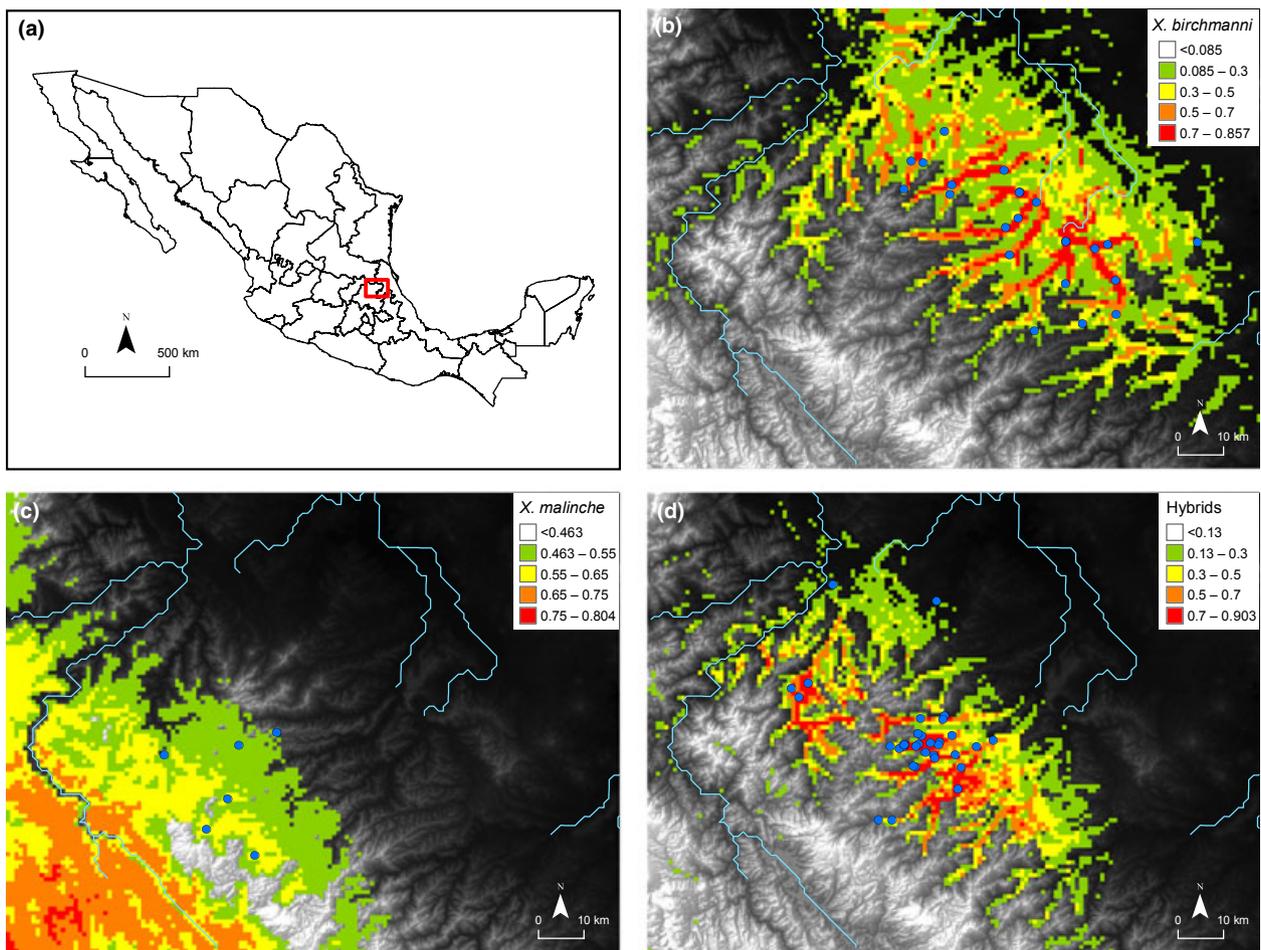


Fig. 1 Map of Mexico with the study area outlined (a). Results of ecological niche modelling (ENM) for *Xiphophorus birchmanni* (b), *X. malinche* (c), and hybrid populations (d). Blue dots indicate the collection localities used to generate the ENMs in Maxent. Colors represent logistic ENM scores (ranging from 0 to 1). Higher values indicate better environmental suitability, and hence, a higher probability for the species to occur.

analysis of each variable's relative contribution to the model and can be used to assess which variables are most important in predicting the species' distribution. Maxent has been shown to perform better than other methods for constructing ENMs, especially when the number of occurrence points is low (e.g. 5–10; Elith *et al.*, 2006; Pearson *et al.*, 2007; Costa *et al.*, 2010). We used autofeatures in Maxent and the default regularization multiplier parameter (1.0). Additionally, we removed duplicate presence points (i.e. points that mapped to the same 1-km² grid cell) and increased the number of iterations to 5000 in order to allow the program to run to the default convergence threshold (10^{-5}).

Although Maxent is able to handle correlation among variables better than other niche modelling methods, reducing the number of variables to those considered ecologically relevant and nonredundant makes testing and interpretation more straightforward (Elith *et al.*, 2011). Further, using fewer variables minimizes the potential for model overfitting (Warren & Seifert, 2011). To reduce the number of variables, we used the principal components tool in the ArcGIS v.9.3 Spatial Analyst extension to construct correlation matrices for our 19 bioclimatic and five hydrographic variables across our spatial extent. For variables that were correlated at $r > 0.9$, we retained only a single variable, preferentially choosing variables that measured extremes over those that measured averages (Shepard & Burbrink, 2008). Extremes (e.g. heat, cold, aridity) were preferentially chosen over averages because they are more likely to set the range limits of species as they provide greater opportunity for selection (Sexton *et al.*, 2009). This procedure left us with 11 variables for use in analyses (eight Worldclim and three Hydro1k; Table S2).

To gauge the sensitivity of each species ENM to the samples used to train the model and to test the predictive ability of the models, we performed 10 replicate runs for each species using a different random seed and subsampling with 66% of samples allotted for training and 34% for testing. For each species, we examined the mean AUC across the 10 replicates and considered a mean AUC value ≥ 0.7 as evidence that the model had sufficient discriminatory ability (Swets, 1988). The final ENM for each species was constructed using all data points to train the model (*X. birchmanni*: $n = 21$, *X. malinche*: $n = 6$, Hybrids: $n = 31$). We overlaid the ENM for each species generated in Maxent on a map of the study region using the Minimum Training Presence threshold to classify grid cells as unsuitable (i.e. outside the species' niche) if they fell below this value (Fielding & Bell, 1997; Liu *et al.*, 2005).

To quantify the amount of niche overlap among species and test whether species occupy different environmental niches, we used a series of measures and tests in the program ENMTools (Warren *et al.*, 2008, 2010). ENMTools uses the ENMs for two species and calculates the degree of niche overlap using two measures: Schoener's *D* and Warren's *I* (see Warren *et al.*, 2008,

2010 for details). For both measures, niche overlap can range from 0, indicating no overlap, to 1, indicating complete overlap. To test whether species occupy different environmental niches, we performed a Niche Identity Test in ENMTools using 1000 iterations (Warren *et al.*, 2008, 2010). Briefly, this test involves multiple iterations of randomizing the locality points for two species, building ENMs in Maxent using the randomized points and then calculating the degree of niche overlap. If the observed niche overlap is $< 95\%$ of the randomized niche overlap values (one-tailed test), then the niches of the two species are considered significantly different (Warren *et al.*, 2008, 2010).

Because niches may be expected to differ in species whose ranges do not overlap greatly, we also used the Background Test in ENMTools to test whether niches are more or less divergent than expected given how environmental conditions vary among species' ranges (i.e. the background environment; Warren *et al.*, 2008, 2010). This test involves multiple iterations in which niche overlap is calculated between one species' ENM and an ENM constructed from random points within the range (i.e. background) of another species. Tests are conducted for all pairwise species comparisons and in both directions. The observed niche overlap is compared to the distribution of overlap values from the runs in each direction to determine whether species' niches are significantly more or less divergent than expected (two-tailed test). Rejection of the null hypothesis indicates that observed niche differences are a function of habitat selection and/or availability, and is often interpreted as evidence for niche conservatism (niches more similar than expected) or niche divergence (niches more different than expected; Warren *et al.*, 2008, 2010). Failure to reject the null hypothesis indicates that niche differentiation between species is explained by variation in the environmental conditions available to each within their respective ranges (Warren *et al.*, 2008, 2010). To delimit species' backgrounds for analysis, we created a minimum convex polygon (MCP) around each species' data points (Warren *et al.*, 2008). The space encompassed by each of these MCPs did not include any area outside the drainages where these species occur, so they contain potentially accessible habitats and should represent suitable backgrounds for analysis (Warren *et al.*, 2008; McCormack *et al.*, 2010). Because fish require aquatic habitats and random points within background areas may sometimes fall in grid cells without such habitats, we were concerned that species niches may appear to be more similar than expected due to their basic requirements for water rather than due to the environmental factors being examined. To address this possibility, we ran Background Tests twice: once with the 11 climatic and hydrologic variables and once with only the eight climatic variables. We ran each Background Test for 1000 iterations.

To further examine the ability of environmental conditions to explain the occurrence of each group and

to identify variables driving niche differentiation, we extracted values for the 11 environmental variables used in ENM from each of our sampling sites and conducted a discriminant function analysis (DFA). The DFA was used to determine the percentage of sites that could be correctly assigned to parental and hybrid populations solely based on environmental conditions. This approach of ENM followed by a multivariate analysis such as DFA provides a more rigorous test of environmental differences among species than either analysis alone can provide (McCormack *et al.*, 2010). ENMs provide a quantitative estimate of each species environmental niche and identify important variables for each species independent of the other species. Because the contributions of variables differ among species ENMs, ENM-based tests for niche differentiation do not reveal the specific environmental factors that differ among species. Using DFA with the same set of variables allows us to test which variables best explain differences in environmental conditions among species and separate their distributions.

Environmental effects on spatial and temporal variation in allele frequencies

Based on our prediction that abiotic environmental conditions will influence the distributions of parentals and hybrids in the *Xiphophorus* system, we asked whether environmental variables that differ between parental species, and potentially limit their distributions could predict spatial and temporal variation in allele frequencies in hybrid populations. To address spatial variation, we used data from one mitochondrial and three unlinked nuclear intron SNPs (Table S3; Culumber *et al.*, 2011) from hybrid populations and subjected genotypic data to a principal components analysis (see Cavalli-Sforza & Feldman, 2003; Patterson *et al.*, 2006). Only one component had an eigenvalue larger than one, and all four markers were highly and positively correlated with each other; that is, the first principal component adequately describes the frequency of *X. birchmanni* and *X. malinche* alleles within a population, with positive scores indicating a surplus of *X. birchmanni* alleles and negative scores a surplus of *X. malinche* alleles (Table S4). We used the results from a DFA of environmental variables separating the two parental species (Table S5a) to determine which variables were most important in discriminating between sites where parental *X. birchmanni* and parental *X. malinche* occur. Based on that discriminant function, five variables (mean temperature of the warmest quarter, minimum temperature of coldest month, maximum temperature of warmest month, precipitation of the driest quarter and mean daily temperature range) with the highest canonical correlations ($r > 0.3$) were used as independent variables in a multiple regression model (based on backwards elimination) to predict variation in *X. birchmanni* allele frequencies across hybrid populations in the SNP data set.

To address temporal variation, we investigated whether temperature variables 90 days prior to the sampling date could predict the incidence of *X. birchmanni* alleles in 10 hybrid populations for which we had samples from more than one point in time. For those 10 hybrid populations, samples had been collected from two to eight different time points between 2003 and 2010 (Table S6). We amplified bi-allelic SNP markers following the methods of Culumber *et al.* (2011) and calculated the minimum temperature, maximum temperature and the mean temperature 90 days before each sampling date based on daily weather data from Tampico, Tamaulipas, Mexico (172 km from the centre of the focal region; obtained at <http://www.wunderground.com>). Allele frequencies (Table S6) were subjected to a principal components analysis as described above. We then subjected the first PC score (indicative of the frequency of *X. birchmanni* alleles in a population) to a repeated-measures linear mixed model, where population was a random factor and minimum temperature, maximum temperature and the mean temperature 90 days before each sampling date (all *z*-transformed) were covariates.

Thermal tolerance

We conducted two thermal tolerance experiments. In the first, we tested wild-caught fish from populations along the elevational gradient. We further wanted to determine whether thermal tolerance is a plastic trait or whether observed differences in thermal tolerance among species were the result of adaptation to local conditions. Therefore, we tested fish that were raised at the CICHAZ field station in a common garden environment in a second experiment (details below).

Experiment 1:

Adult fish were collected using baited minnow traps, seines or dip nets from 11 sites along a continuum from high to low elevation (Table S1). Stream temperature was recorded at each sampling locality using a standard glass thermometer. Fish were transported alive to the CICHAZ field station and kept in thermally insulated coolers until testing (1–2 h) to maintain water temperature as close to the temperature at the collection site as possible. All fish were tested ($N = 496$; Summer $N = 307$, Winter $N = 189$) on the same day that they were collected, and the experiment was repeated twice for each population: once in June 2007 (summer) and again in December 2007/January 2008 (winter). Sample sizes were smaller for the winter season because water flow is greater and temperatures are lower during the winter in this region, making fish less active and more difficult to collect.

Experiment 2:

In order to test whether variation in thermal tolerance is due to local adaptation or phenotypic plasticity, we

reared fish in a common garden environment in concrete stock tanks ($1 \times 1 \times 2$ m) with a water flow-through system at a common elevation of 981 m at the CICHAZ field station. Forty-eight gravid females of each parental species were collected from Chicayotla for *X. malinche* and Coacuilco for *X. birchmanni*. Those 48 females were divided evenly among 16 concrete tanks such that each of eight tanks received six gravid *X. malinche* and eight tanks received six gravid *X. birchmanni*. Females were allowed to give birth in the tanks, and fry were reared out with minimal interference other than to supplement food in the first months until natural fauna and flora such as algae began to develop and provide a food source. The fish were allowed to mature for a period of 1 year and then were tested in thermal tolerance trials. As above, the temperature of the water in the stock tanks was measured prior to testing. From each of the 16 stock tanks, we tested one male and one female in hot trials and one male and one female in cold trials. For *X. malinche*, we tested a total of 32 fish: 16 (8M: 8F) in hot and 16 (8M: 8F) in cold. The same numbers of *X. birchmanni* fish were tested except that only seven females were tested in hot for a total of 31 fish. To avoid potential researcher bias, these trials were carried out blindly such that the observer did not know which species were in which stock tanks.

In all thermal tolerance trials, a HOBO temperature logger (Onset Corporation) was submerged in the hot and cold test tank to obtain an exact reading of temperature throughout each trial. For hot trials, an enamel container containing a 4 L of water and the test fish was placed onto an inverted ceramic plate inside a larger enamel container. The apparatus was suspended over a gas burner and heated at a rate of 0.3 °C per min. In cold trials, fish were placed in a standard volume of water in a plastic container and frozen gel packs were placed in the water to reduce the temperature by 0.3 °C per min. Water temperature at initial loss of equilibrium (ILOE), analogous to the critical thermal maximum and minimum, was recorded for each individual in all trials. Initial loss of equilibrium is the time at which a fish begins to lose its ability to right itself. At final loss of equilibrium, when the fish had lost all ability to maintain its balance and stayed on its side for more than one full second, we removed it from the trial and placed it in a recovery tank. For Experiment 1, we used ANOVA to test for the effects of species, population nested within species, season and the interaction term species \times season on cold and heat tolerance. For Experiment 2, we used ANOVA to test for the effects of species and sex on the dependent variables cold and heat tolerance.

***hsp* expression and thermal stress**

For the experiment examining *hsp* expression, we collected *Xiphophorus birchmanni* from the locality Garces on the Río Garces, *X. malinche* from Chicayotla on the Arroyo

Xontla and hybrids from Calnali-mid on the Río Calnali (see Table S1). Specimens for baseline gene expression were collected in the wild and preserved whole in RNAlater (Life Technologies Corporation, Carlsbad, CA, USA). Quantification of baseline *hsp* expression in wild-caught fish has been previously described (Coleman *et al.*, 2009), and relative expression following thermal stress in the laboratory followed the same methods.

To characterize the regulation of *hsp* response, four *X. birchmanni*, four *X. malinche* and four hybrids were subjected to an acute heat stress followed by qPCR. All fish had been collected in the wild and housed in 23 °C water for 14 months, and then, those same fish were subjected to the heat stress experiment. We stressed fish by putting them individually in an Erlenmeyer flask with 1000 mL of water at 23 °C, placing the flask above a lit Bunsen burner and heating the water to 33 °C at a rate of 1 °C per min. We killed fish immediately following and placed their heads in RNAlater until RNA isolation, cDNA synthesis and qPCR (methods followed Coleman *et al.*, 2009) using QuantumRNA Universal 18s rRNA (Ambion, Inc.) as an internal reference gene to normalize each sample. We used ANOVA to test for differences in mean baseline *hsp* expression among populations, as well as mean relative *hsp* expression following acute heat stress. When a significant difference ($P < 0.05$) was observed, Fisher's LSD was used to test for differences between populations with Bonferroni correction for multiple comparisons.

Results

Ecological differentiation and niche modelling

Across the 10 subsampling replicates, the ENMs for all species produced mean test AUC values >0.7 (*X. birchmanni*: mean = 0.923, SD = 0.028; *X. malinche*: mean = 0.748, SD = 0.142; hybrids: mean = 0.940, SD = 0.017), indicating a sufficient ability to discriminate between presence and absence locations (Swets, 1988). In the final ENMs, species differed in which of the 11 variables contributed most to their model (Table S2). For *X. malinche*, minimum temperature of the coldest month (61.6%; relative contribution to Maxent model) and mean daily temperature range (36.3%) contributed the most, whereas flow accumulation (31.9%), precipitation of the driest quarter (22.0%) and minimum temperature of the coldest month (15.7%) had the highest contributions for *X. birchmanni*. For hybrids, the ENM was described primarily by annual temperature range (29.7%), mean diurnal temperature range (16.2%) and precipitation of the driest quarter (14.8%). The predicted distributions of *X. birchmanni* and hybrids fit relatively well with their actual distributions; however, the ENM of *X. malinche* predicts that the area where it occurs is marginally suitable and that environmental conditions are more optimal at higher elevations to the south-west (Fig. 1). However, *X. malinche* are not known to occur in

that area and the fact that they occur farther north-west at slightly lower elevation than predicted by the ENM suggests that physical barriers to dispersal such as waterfalls may have prevented historical upstream migration. The small number of locality points for this narrowly distributed species ($N = 6$) may also limit the ability of ENM methods to predict the species distribution with high precision.

Niche identity tests showed that both parental species and hybrids occupy significantly different niches. Niche overlap between the parentals was significantly less than expected from random [Schoener's $D = 0.282$, $P < 0.001$; Warren's $I = 0.560$, $P < 0.001$]. Likewise, niche overlap between hybrids and parentals was significantly less than expected by chance [to *X. birchmanni*: $D = 0.358$, $P < 0.001$; $I = 0.625$, $P < 0.001$; to *X. malinche*: $D = 0.392$, $P = 0.001$; $I = 0.710$, $P = 0.007$]. Background tests indicate that the niche of *X. birchmanni* is more similar to the niche of *X. malinche* than expected by the background environment where *X. malinche* occurs ($P = 0.014$ for both D and I). However, the niche of *X. malinche* is more divergent from the niche of *X. birchmanni* than expected by the environmental background where *X. birchmanni* occurs, although significance was marginal ($P = 0.092$ for D , $P = 0.046$ for I). This seemingly counterintuitive result can be interpreted as both species having similar environmental preferences, but the availability of those preferred environmental conditions is limited within the range of the species showing greater differentiation than expected, which in this case is *X. malinche* (Nakazato *et al.*, 2010). Niches of *X. birchmanni* and the hybrids are no more or less divergent than expected based on their respective background environments (hybrid vs. *X. birchmanni* background: $P = 0.614$ for D , $P = 0.262$ for I ; *X. birchmanni* vs. hybrid background: $P = 0.312$ for D , $P = 0.116$ for I). The niche of hybrids was neither more nor less divergent from the niche of *X. malinche* than expected by the background environment of *X. malinche* ($P = 0.308$ for D , $P = 0.304$ for I); however, the niche of *X. malinche* was more divergent from the niche of hybrids than expected by the environmental background where hybrids occur ($P = 0.002$ for D , $P = 0.012$ for I). Again, this result may be due to limited availability of the preferred environmental conditions within the range of *X. malinche*. Results of niche identity and background tests were similar when analyses were run without the hydrologic variables (results not shown).

Using values of environmental variables extracted from each site, DFA indicated that 90.5% of the sites (compared to 33% based on random expectations) could be classified correctly as *X. malinche*, *X. birchmanni* or hybrid based on environmental conditions (Table S5b). Function 1 essentially separates *X. birchmanni* sites (positive scores) from *X. malinche* sites (negative scores), with hybrids being intermediate. Function 2 separates hybrid (positive scores) and *X. malinche* sites (negative scores), with

X. birchmanni sites being intermediate. Temperature variables were the strongest variables separating species and contributing to the classification of sites.

Environmental effects on spatial and temporal variation in allele frequencies

The same abiotic environmental factors that differentiate habitats occupied by *X. birchmanni* and *X. malinche* also predicted frequencies of *X. birchmanni* alleles across hybrid populations (Table 1, Fig. 2). The only factor retained in the final multiple regression was the minimum temperature of the coldest month. The positive correlation of *X. birchmanni* allele frequencies with minimum temperature of the coldest month was consistent with *a priori* expectations based on the DFA.

Temperature variables also were instrumental in predicting parental allele frequencies in hybrid populations through time. As expected from the previous analysis, we found a significant effect of population on allele frequency ($P = 0.038$; see Table 2 for details). In addition, minimum temperature in the previous 90 days was significantly related to allele frequencies ($P = 0.005$; Fig. 3), whereas maximum temperature was marginally nonsignificant ($P = 0.062$). Mean temperature in the past

Table 1 Multiple regression model (based on backwards elimination) predicting the frequency of *Xiphophorus birchmanni* alleles in hybrid populations based on local abiotic environmental factors ($R^2 = 0.562$, ANOVA: $F_{1, 20} = 8.793$, $P = 0.008$).

	B	SE	Beta	<i>t</i>	<i>P</i>
Constant	-0.009	0.190		-0.046	0.964
Minimum temperature of coldest month	0.576	0.194	0.562	2.965	0.008

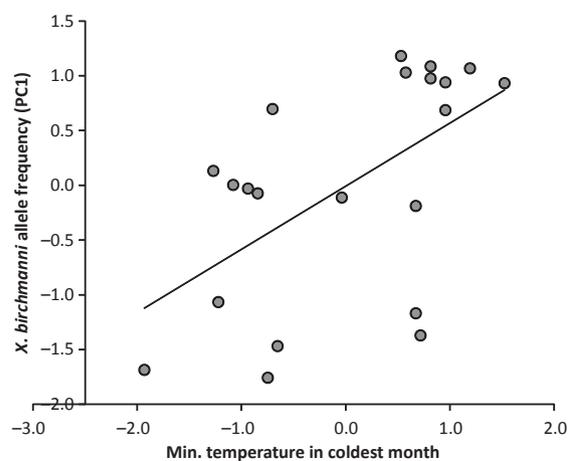


Fig. 2 Correlations between minimum temperature in the coldest month and *Xiphophorus birchmanni* allele frequencies in hybrid populations. The *x*-axis represents *z*-transformed temperature values ($^{\circ}\text{C}$) and the *y*-axis allele frequencies derived from principal component analysis.

Table 2 Results of linear mixed model to assess the effects of minimum, maximum and average temperature on temporal variation in allele frequencies.

Estimates of fixed effects	Estimate	SE	d.f.	<i>t</i>	<i>P</i>
Intercept	0.219	0.308	9.047	0.713	0.494
Minimum temperature in past 90 days	-0.311	0.094	14.620	-3.323	0.005
Maximum temperature in past 90 days	-0.117	0.047	4.489	-2.468	0.062
Average temperature in past 90 days	0.065	0.076	9.389	0.855	0.414
Estimate of covariance parameters	Estimate	SE	Wald	Z	<i>P</i>
Population	0.924	0.446	2.071	0.038	

90 days had no significant relationship with allele frequency ($P = 0.414$).

Thermal tolerance

Experiment 1:

In wild-caught fish, both heat and cold tolerance varied significantly among populations along an elevational gradient (Fig. 4). ANOVA for cold tolerance showed significant effects of season ($F_{1,2} = 24.323$, $P = 0.038$) and population nested within species ($F_{9,227} = 13.142$, $P < 0.001$). The interaction of species \times season was significant ($F_{2,227} = 8.374$, $P < 0.001$), indicating a difference in cold tolerance among species but in only one season. Indeed, post hoc, least significant difference (LSD) tests showed that all pairwise comparisons of species' cold tolerance were significant ($P < 0.001$). Full tables for cold and heat tolerance ANOVAs are shown in Table S7. The effect of season stemmed from the fact that all species had lower cold tolerance in the winter than in the summer, but *X. birchmanni* and hybrids had larger seasonal downshifts, 3.70 and 2.93 °C, respectively, whereas *X. malinche* only decreased 2.16 °C. ANOVA on heat tolerance also showed significant effects of season ($F_{1,2} = 54.421$, $P = 0.017$) and population nested within species ($F_{9,239} = 19.306$, $P < 0.001$). The significant interaction of species \times season ($F_{2,239} = 6.593$, $P = 0.002$) again indicated significant species differences in heat tolerance in one season and post hoc LSD comparisons demonstrated that heat tolerance was significantly different in all species comparisons ($P < 0.001$). The significant interaction of species and season appeared to be due to a smaller seasonal downshift in heat tolerance in *X. birchmanni* (2.78 °C) compared to *X. malinche* and hybrids (4.54 and 4.56 °C, respectively). Temperatures of critical thermal maxima and minima in this study correspond to biologically relevant temperatures (GGR unpublished data). Data recorded from underwater temperature loggers have shown lows of 6 °C at high elevations typical of *X. malinche* (~1100 m) and highs of 42 °C at an *X. birchmanni* locality (~200 m).

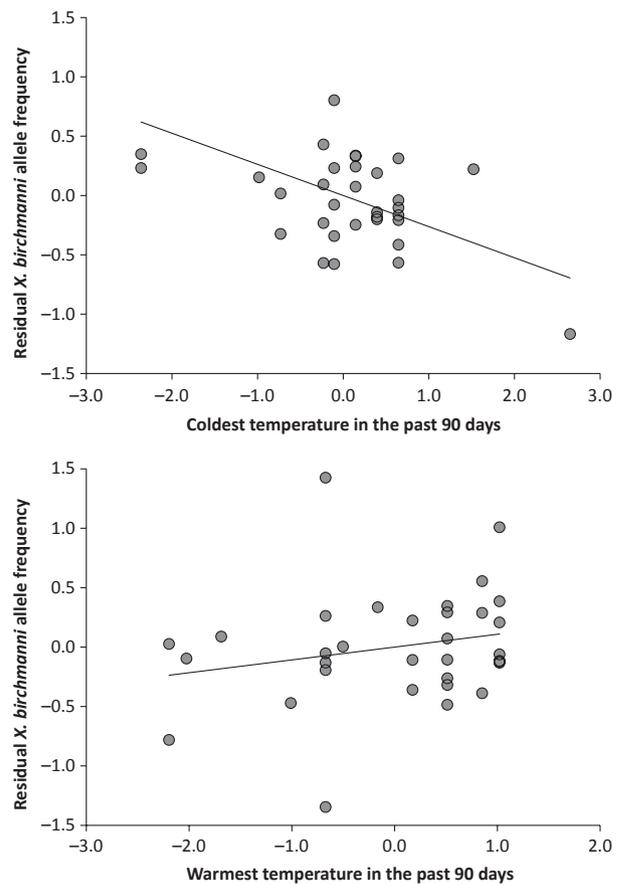


Fig. 3 Temporal variation in *Xiphophorus birchmanni* allele frequency in relation to different temperature variables. Plotted are residuals based on ANOVA that removed variation among populations. The x-axis of both figures represents z-transformed temperature values (°C) and the y-axis allele frequencies derived from principal component analysis.

Experiment 2:

Critical thermal maximum but not critical thermal minimum was significantly different between species raised in the common garden. In hot trials, *X. birchmanni* exhibited significantly greater heat tolerance than *X. malinche* (*X. birchmanni*, mean = 36.15 °C; *X. malinche*, mean = 35.48 °C; $F_{1,30} = 6.829$, $P = 0.014$). There was no difference between sexes ($F_{1,30} = 0.140$, $P = 0.711$). In cold tolerance trials, there was no significant difference between species ($F_{1,30} = 0.139$, $P = 0.712$) nor sexes ($F_{1,30} = 0.014$, $P = 0.905$).

hsp expression and thermal stress

When analyses of thermal tolerance were narrowed to only those populations used for *hsp* and thermal stress experiments, significant effects of species and season on both cold and heat tolerance were present (data not shown). For reference, the *X. birchmanni*, *X. malinche* and

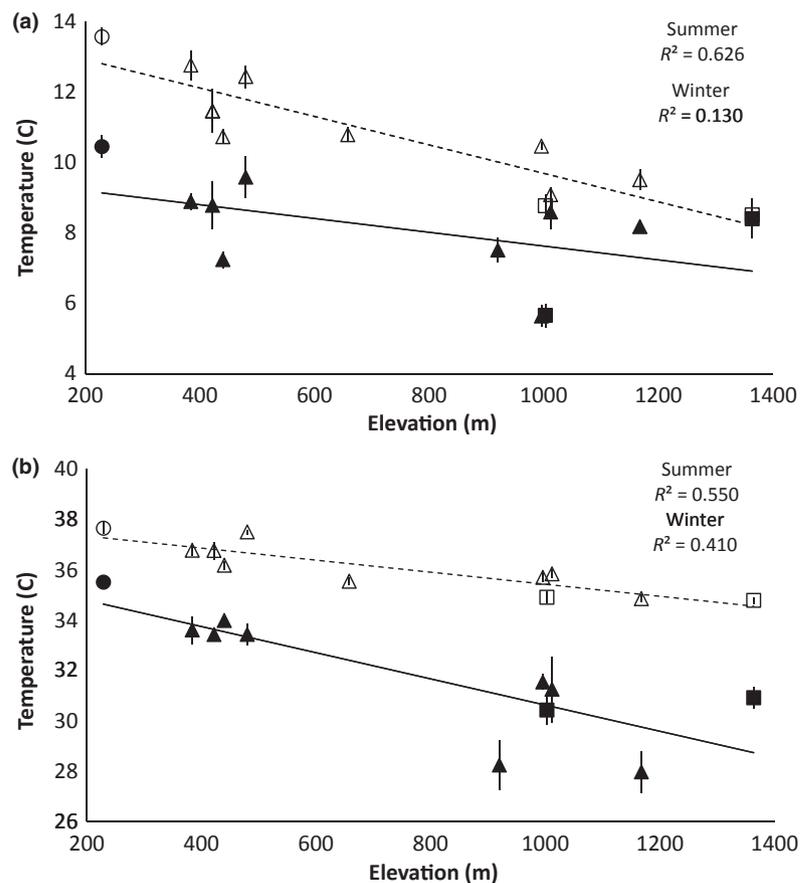


Fig. 4 (a) Critical thermal minima (\pm SE) along an elevation gradient observed in the summer (open symbols, dotted line) and again in the winter (closed symbols, solid line). Species are designated with different symbols (\circ : *Xiphophorus birchmanni*, \square : *X. malinche*, \triangle : hybrid). (b) Critical thermal maxima (\pm SE) observed in the summer (open symbols, dotted line) and winter (closed symbols, solid line).

hybrid populations used for *hsp* and thermal stress analyses are denoted in Fig. 4a,b. The upstream-to-downstream patterns in thermal tolerance among these three populations mirrored that observed over the entire elevational gradient.

Baseline *hsp* expression levels of wild-caught individuals were significantly different among the three groups (*hsp*70: $F_{2,9} = 5.46$, $P = 0.03$; *hsp*90: $F_{2,9} = 6.44$, $P = 0.02$) and showed a pattern identical to that observed for thermal tolerance: *X. birchmanni* exhibited the highest expression, *X. malinche* the lowest, and hybrids were intermediate (Fig. 5). Following acute thermal stress experiments, we again found significant differences in *hsp* expression (*hsp*70: $F_{2,9} = 82.63$, $P < 0.0001$; *hsp*90: $F_{2,9} = 60.93$, $P < 0.0001$), but not in the same pattern of baseline expression. Instead, hybrids showed up-regulation of *hsps* equivalent to that of *X. birchmanni*, whereas *X. malinche* showed a relatively weak *hsp* response (Fig. 5).

Discussion

Physiological adaptation along an elevational gradient appears to play a key role in producing replicated structure in the *birchmanni*–*malinche* hybrid zones. The parental species, *X. birchmanni* and *X. malinche*, as well as

their hybrids exhibited distinct ecological niches with abiotic environmental factors, in particular temperature variables, driving niche differences among groups. More interestingly, thermal tolerance of parentals and hybrids, which is mediated in part by expression of genes encoding heat-shock proteins (Feder & Hoffmann, 1999), matches their respective distribution patterns (*X. birchmanni*: low elevation, *X. malinche*: high elevation, hybrids: intermediate). Taken together this suggests that, in the face of historical physiological adaptation to different thermal conditions in parentals, the elevational gradient provides a conduit for secondary contact between the species, with hybrids experiencing equal or greater fitness in intermediate thermal environments. This study represents a major step towards understanding the physiological and genetic bases of a fitness-related trait that affects hybrid zone structure in the swordtail system. More broadly, these results build upon an understudied theme in evolutionary biology, namely understanding the role and importance of thermal adaptation in the emergence of reproductive isolation (Keller & Seehausen, 2012).

A weakness of correlative approaches, such as ENM, is the potential for results to be overinterpreted – as correlated environmental variables that are not actually

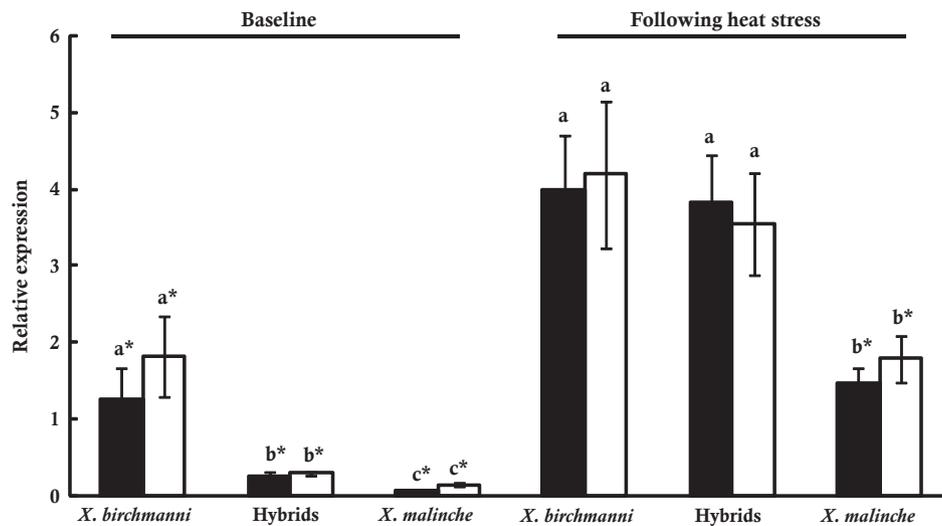


Fig. 5 Baseline relative expression levels of wild-caught fish and relative expression (mean \pm SE) following acute thermal stress in the lab of *hsp70* (black bars) and *hsp90* (white bars). Within genes and experiments, bars with different letters above them are significantly different from one another; asterisks indicate differences in expression between species within treatment where $P < 0.05$ after Bonferroni correction.

used in the analysis might be the variables driving the observed patterns in the data. However, integrating mechanistic and correlative approaches, as we did here, provides a means to confirm whether variables used in the correlative analyses actually have direct and relevant effects on the focal organism. In this study, we used ENM techniques and correlations between environmental variables and allele frequencies to identify temperature as an important variable and then confirmed through mechanistic experiments and the quantification of gene expression that temperature does indeed have significant effects on organism performance.

Niche differentiation and the abiotic environment

Parental *X. birchmanni* and *X. malinche* occur at opposite ends of an elevational gradient, and hybrids dominate at intermediate elevations (Rosenthal *et al.*, 2003; Culumber *et al.*, 2011), suggesting that this pattern is maintained by adaptation to local conditions along the environmental gradient. The use of ENM in other systems, combining geo-referenced species occurrence data with spatially explicit data on abiotic and/or biotic variables (reviewed in Kozak *et al.*, 2008), has proven useful for delineating species distributions (Raxworthy *et al.*, 2007; Shepard & Burbrink, 2008, 2011; McCormack *et al.*, 2010), but is in its infancy in dissecting the basis of hybrid zone structure (Cicero, 2004; Swenson, 2006; Chatfield *et al.*, 2010). Here, ENM enabled us to disentangle the complexity of environmental differences along the elevational gradient. As with many environmental gradients, stream gradients are multivariate, consisting of superimposed and correlated abiotic and biotic variables, such as temperature, precipitation,

food availability, vegetation and predation (Endler, 1995; Grether *et al.*, 1999; Kolluru *et al.*, 2007; Walsh & Reznick, 2009). Using ENM followed by DFA allowed us to identify abiotic factors important in predicting the distribution of parentals and their hybrids, and helped us to focus on fitness-relevant variables that were the strongest drivers of the observed patterns.

Among factors included in our analysis that vary along the elevational gradient, temperature variables emerged as the strongest predictors of hybrid zone structure at multiple levels of analysis. Temperature plays a critical role in determining plant (C4 grasses, Edwards & Still, 2008; aquatic macrophytes, Sawada *et al.*, 2003) and animal distributions (*Myotis* bats, Humphries *et al.*, 2002; land birds; Root, 1988; zooplankton, Southward *et al.*, 1995) and can drive local adaptation within species (Miller & Packard, 1977; Feminella & Matthews, 1984; Ayres & Scriber, 1994). Moreover, temperature may be an important factor in structuring other hybrid zones, yet few studies have considered the multidimensionality of temperature variation and instead have focused mostly on mean temperature (Swenson, 2006; Cheviron & Brumfield, 2009). Our data suggest that extreme temperature events are likely to have more important physiological and survival consequences than mean temperature. Similarly, short-term exposure of corals and associated sea anemones to above or below average temperatures significantly increases loss of symbiotic zooxanthellae, accelerating bleaching (Steen & Muscatine, 1987; Jokiel & Coles, 1990).

Temperature variables were good predictors for broad spatial patterns in the occurrence of *Xiphophorus*, contributing not only to the correct classification of more than 90% of sites as pure *X. birchmanni*, pure *X. malinche*

or hybrid based on environmental conditions, but also to the successful prediction of allele frequencies in hybrid populations. Correlations between parental allele frequencies and minimum temperature in the coldest month matched the *a priori* prediction based on parental species niche differentiation. Moreover, temperature variables were able to explain temporal variation in species-specific allele frequencies in hybrid populations, indicating that even at a finer scale within hybrid populations, temperature was still able to predict variation in population genetic structure. Correlations between temporal variation in allele frequencies and temperature revealed a significant and marginally non-significant effect of minimum and maximum temperature, respectively, but no effect of mean temperature. One interpretation that we cannot entirely rule out is that fish respond behaviourally, shifting their distribution in response to seasonal changes in temperature. This is not well supported by the data given that mean temperature, the best predictor of seasonal temperature change, was unrelated to allele frequencies. Rather, the data suggest that extreme, short-term changes in temperature serve as selective events and are sufficient to significantly alter the structure of hybrid populations leading to long-term maintenance of hybrid zone structure.

Selection on thermal tolerance

Thermal tolerance is a performance metric of the ability to buffer against a wide range of negative, temperature-dependent physiological and behavioural changes that can directly impact an individual's fitness (Donaldson *et al.*, 2008 and references therein). The dominant role of temperature in explaining the spatial structure of the *birchmanni*–*malinche* hybrid zones suggested that animals should be adapted to local thermal conditions along the elevational gradient. Indeed, along the gradient, both species and population significantly explained performance in thermal tolerance experiments for both heat and cold tolerance. *X. birchmanni* and *X. malinche* performed best in warmer and cooler temperatures, respectively. However, hybrids outperform both parental counterparts at intermediate elevations because they exhibit greater cold tolerance than *X. birchmanni* and greater heat tolerance than *X. malinche*. These results are qualitatively similar to those found in an intertidal copepod, *Tigriopus californicus*, which face trade-offs between thermal tolerance at extreme temperatures and competitive ability at moderate temperatures (Willet, 2010). Taken together, these results have important implications for hybrid zones occurring along environmental gradients. For example, in our system, each parental species exhibits better performance at their respective end of the elevation spectrum, but underperforms hybrids at intermediate elevations where temperatures are more moderate. When extreme temperatures do occur at intermediate elevations where hybrid pop-

ulations are found, species-specific allele frequencies responded accordingly with decreases in *X. birchmanni* alleles following cold events and decreases in *X. malinche* alleles following hot events. Furthermore, variation in long-term averages of climatic variables at hybrid sites is mirrored by changes in parental allele frequencies.

Importantly, *X. birchmanni* and *X. malinche* reared under identical environmental conditions at a common elevation still exhibited significant differences in heat tolerance. This indicates that phenotypic plasticity alone cannot explain the variation in thermal tolerance observed along the elevational gradient. This is further supported by the significant temporal relationship between temperature and allele frequencies. Cold tolerance, while different in wild-caught parentals, did not differ between parentals reared in the common garden. One potential explanation for this is the unusually cold temperatures that common garden fish experienced during rearing in the winter of 2010 prior to our experiments. Temperatures reached as low as 13 °C for several days, and increased mortality was observed in *X. birchmanni* stock tanks compared to *X. malinche* tanks during this period. Thus, the cold temperatures likely represented a strong selective event against the least cold-tolerant *X. birchmanni*, thereby leaving the most cold-tolerant survivors. This is consistent with observations that allele frequencies in hybrid populations fluctuate according to the temperature in the previous 90 days and seems more plausible than assuming that cold tolerance is plastic whereas heat tolerance is not, given that they are under the control of the same family of genes. Regardless of what might explain this result in the present study, it is nonetheless consistent with other findings. For example, Barrett *et al.* (2011) observed a significant variation in cold tolerance between marine and freshwater sticklebacks but no difference in their heat tolerance.

Thermal tolerance and *hsp* gene expression

Thermal tolerance depends in part on the expression of *hsps* (Feder & Hoffmann, 1999). Elucidating the genetic underpinnings of adaptive traits contributing to fitness has long been a goal of evolutionary biologists (Lewontin, 1974) and investigations into the genetics of hybridization have yielded many insights into the role of particular genes or interactions of genes that contribute to sterility, incompatibility and increased/reduced fitness of hybrids (for review, see Burke & Arnold, 2001). Few studies, however, have addressed how genetic mechanisms of superior hybrid fitness interact with specific environmental variables, which should play a deterministic role in structuring hybrid zones. Baseline gene expression of *hsps* closely matched thermal tolerance with hybrids intermediate to the parental species. *Xiphophorus malinche* showed a significantly weaker *hsp* response to acute heat stress than did *X. birchmanni* or hybrids. The significantly weaker *hsp* response, and

overall lower *hsp* expression of *X. malinche*, suggests that it is confined to cooler waters with relatively stable thermal profiles, which is supported by ENM results. Indeed, *X. malinche* has a restricted distribution and is known from only six headwater populations (Rauchenberger *et al.*, 1990; Rosenthal *et al.*, 2003; Culumber *et al.*, 2011). Although the patterns in *hsp* response closely matched what would be predicted based on results from ENM, spatial and temporal analyses and thermal tolerance trials, future replication would be ideal in order to confirm this pattern.

Plasticity and adaptation are two potential explanations for the significant correspondence between thermal tolerance (and *hsp* expression) and hybrid zone structure. The first, which we can largely rule out, is the extent to which thermal tolerance and *hsp* gene expression are plastic traits throughout life or may have an ontogenetic component. Our common garden experiment, rearing each parental under identical environmental conditions, demonstrated that critical thermal maxima were still significantly different between species. Additionally, several lines of evidence indicate that there is heritable variation in thermal tolerance among groups. For example, differences in *hsp* expression persisted in fish maintained under identical conditions in the laboratory for over a year. Given the robust ENM results, strong correlation between temperature variables and allele frequencies and maintenance of a significant difference in heat tolerance in the common garden, adaptation to the thermal environment along the elevational gradient is the most plausible explanation.

Temperature gradients and the maintenance of hybrid zone structure

In a recent literature review, Keller & Seehausen (2012) suggest that abiotic gradients, specifically thermal environments, may be a common driver of ecological speciation, but that it is an understudied area of research. In fact, they point out that the effect of thermal gradients might be particularly pronounced in aquatic environments of tropical mountain ranges and that such scenarios may provide opportunities for hybrids to outperform parentals in intermediate locations along the gradient. Our results indicate that the *birchmanni-malinche* hybrid zones, which are structured along a thermal gradient in an aquatic habitat of a neotropical mountain range, represent an example of the scenario they predict.

Two alternatives to bounded hybrid superiority are globally superior hybrids (hybrid swarm) and globally less-fit hybrids (tension zone). If hybrids were globally more fit than parentals, then hybrid zone structure would quickly erode creating a hybrid swarm and 'extinction by hybridization' of parentals. Hybrids have occurred since at least the late 1990s yet remain restricted to intermediate elevations in seven different

stream reaches with pure parental populations remaining intact at either end of the hybrid zones (Rosenthal *et al.*, 2003; Culumber *et al.*, 2011). On the other hand, if hybrids are globally less fit, then hybrid zone structure could only be maintained with recurrent hybridization between pure parentals to produce new F_1 offspring. With 8 years of genotyping data from this study and a previous study, including >500 individuals from populations where both pure parentals occur together, fewer than 2% have had F_1 genotypes (Culumber *et al.*, 2011). This is below expectations based on random chance and given misclassification rates based on the number of markers used.

Instead, data from a number of studies suggest that at least a subset of hybrid males are preferred by females due to transgressive segregation in male secondary sex traits (Rosenthal *et al.*, 2003; Rosenthal & Garcia de Leon, 2006; Fisher *et al.*, 2009). The present data show that intermediate elevations are too warm for *X. malinche* and too cold for *X. birchmanni* leading to superior performance by hybrids within the intermediate zone. Conversely, hybrids are bounded due to the fact that parental *X. malinche* and *X. birchmanni* outperform hybrids at high and low elevations, respectively, and that hybrids underperform in more extreme cold and hot temperatures. The hybrid zones are replicated across seven separate stream reaches (Culumber *et al.*, 2011) and, as shown herein, the zone of abiotic conditions in which hybrids occur is also replicated in each of these stream reaches. Thus, the *birchmanni-malinche* hybrid zones more closely fit a model of bounded hybrid superiority than alternative models. This is consistent with thermal structuring along another elevational gradient in *Drosophila*, where a high- and low-elevation species are each best adapted to different temperature regimes which contributes to premating isolation and prevents significant gene flow along the gradient (Matute *et al.*, 2009).

Conclusion

Hybrid zones often coincide with variation in abiotic or biotic factors, and there is evidence that selection via particular abiotic variables contributes to hybrid zone structure and fitness differences (Nikula *et al.*, 2008; Cheviron & Brumfield, 2009). Such studies provide a basis to identify specific agents of selection and then explicitly test the mechanisms involved. Yet, few studies have measured the effect of selective agents on individual performance and physiology across a hybrid zone. Our study demonstrates the power of using an integrative approach not only to identify variables that may underlie hybrid zone structure, but to then directly test performance and physiological response to relevant variables. The results of this approach indicate that natural selection based on thermal regimes, and possibly other factors correlated with elevational variation in temperature,

stabilizes the replicated hybrid zones in this system. Adaptive physiology is an understudied but important determinant of hybrid zone structure and, more broadly, of the evolutionary outcomes of natural hybridization. Physiological and behavioural traits have long been neglected in the study of adaptive hybridization in animals, largely due to a lack of integration of laboratory and field studies. Future studies of how environmental variation and hybridization interact to affect adaptive processes will continue to aid in our understanding of the evolution of biodiversity from both ecological and physiological perspectives.

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Supporting information

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

Table S1 List of collection sites.

Table S2 Environmental variables used to construct ENMs and estimates of the relative contribution of each variable (%) to the Maxent model for each species.

Table S3 *X. birchmanni* allele frequencies in different hybrid populations from SNP markers genotyped in Culumber *et al.* (2011).

Table S4 Principal components on *X. birchmanni* allele frequencies in hybrid populations at four SNP loci.

Table S5 Discriminant function analysis to differentiate *X. malinche*, *X. birchmanni*, and hybrid sites based on environmental conditions.

Table S6 *X. birchmanni* allele frequencies in hybrid populations used for temporal analyses.

Table S7 (A) Full table of ANOVA for cold tolerance along the elevational gradient. (B) Full table of ANOVA for heat tolerance.

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