

Environmental variation, hybridization, and phenotypic diversification in Cuatro Ciénegas pupfishes

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transgressive segregation.

Abstract

Hybridization can generate novel phenotypes, and in combination with divergent selection along environmental gradients, can play a driving role in phenotypic diversification. This study examined the influence of introgressive hybridization and environmental variation on the phenotypic diversity of two pupfish species (*Cyprinodon atrorus* and *Cyprinodon bifasciatus*) endemic to the Cuatro Ciénegas basin, Mexico. These species occupy opposite environmental extremes and are comprised of multiple, intraspecifically isolated populations. However, interspecific hybridization occurs to various degrees within connecting, intermediate environments. Using geometric morphometric analysis, extensive variation of body shape was observed between and within species, and phenotypic variation was strongly correlated with environmental conditions. Furthermore, some introgressed populations exhibited unique phenotypes not found in either of the parents, and overall morphospace occupation was significantly higher in introgressed populations when compared to the parentals. Overall, we find environmental variation and transgressive segregation both appear to have been important in shaping phenotypic variation in this system.

Introduction

Understanding the origins of biodiversity requires basic knowledge of at least two fundamental types of evolutionary mechanisms: those leading to phenotypic diversification and those underlying the evolution of reproductive isolating barriers. These contribute to different aspects of diversity and can operate independently. Phenotypic divergence, which is typically driven by natural selection, leads to functional diversity and, in absence of reproductive isolation, to intraspecific variation and polymorphisms (e.g. in trophic morphology: Hulsey *et al.*, 2005; in colouration: Maan *et al.*, 2006). The evolution of reproductive isolation, on the other hand, leads to species diversity, but not necessarily phenotypic divergence. Consequently, the evolution of reproductive isolation can give rise to complexes of

cryptic species (Evans *et al.*, 2008; Murphy *et al.*, 2009). The two processes, however, often act in concert and can directly affect each other. Mounting evidence indicates that reproductive isolation can evolve as a by-product of adaptive trait divergence (ecological speciation: Schluter, 2001; Rundle & Nosil, 2005; Nosil *et al.*, 2009). At the same time, the break down of reproductive isolation – i.e. hybridization between distinct lineages – can have profound impacts on phenotypic evolution and lead to the emergence of novel adaptations through trait recombination (Lewontin & Birch, 1966; Seehausen, 2004; Parnell *et al.*, 2008).

Although hybridization has been long known to play an important role in plant diversification and speciation (Grant, 1981; Hegarty & Hiscock, 2005; Soltis & Soltis, 2009), its role in animal diversification is much more controversial and has often been found to be negligible (Mayr, 1963; Coyne & Orr, 2004; Abbott *et al.*, 2008). With the wider application of molecular genetics techniques, however, a high prevalence of introgression and numerous occurrences of homoploid hybrid speciation have been documented in animals (e.g. Salzburger *et al.*,

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2002; Seehausen *et al.*, 2003; Schliewen & Klee, 2004; Gompert *et al.*, 2006; Mallet, 2007; Schwarz *et al.*, 2007; Jiggins *et al.*, 2008). These recent findings have not only helped affirm the evolutionary importance of hybridization, but have also inspired novel arguments that address the mechanisms involved, such as the hybrid swarm theory of adaptive radiation (Seehausen, 2004). This theory posits that admixture can drive functional diversification during adaptive radiations when distinct lineages invade new environments and hybridize. This is thought to occur through the creation of new adaptive trait combinations and the generation of novel phenotypes, whereby hybrid offspring exhibit trait distributions outside of the parental range (transgressive segregation). Empirical studies indicate that the effects of transgressive segregation are positively correlated with the genetic distance of the parental species (Stelkens & Seehausen, 2009), and there is evidence for a potential role of introgression and hybridization in phenotypic diversification from experimental and field studies (Riesenberg *et al.*, 1999; Albertson *et al.*, 2003; Rosenthal *et al.*, 2003; Albertson & Kocher, 2005; Herder *et al.*, 2006; Gardner & Latta, 2008).

Novel phenotypes can, of course, be generated through transgressive segregation, simple mutation, or recombination of existing genetic variation. But to evolutionarily persist, a given phenotype must prove selectively advantageous under the environmental conditions in which it occurs. This results in predictable patterns of phenotypic variation that match particular environmental conditions, and such correlations between environments and phenotypes have been documented across a wide spectrum of taxa and spatial scales (e.g. Jimenez-Ambriz *et al.*, 2006; Manier *et al.*, 2007; Fontanier & Tobler, 2009; Silva *et al.*, 2010). Consequently, a fundamental question in evolutionary biology is how forces generating novel phenotypic variants, in conjunction with selection based on environmental conditions, shape phenotypic variation in natural systems.

This study addressed the influence of environmental variation and introgressive hybridization on the phenotypic diversification of two naturally hybridizing pupfishes endemic to the Cuatro Ciénegas basin of the Chihuahuan Desert in Mexico. Pupfishes are small-bodied fish of the genus *Cyprinodon* (Cyprinodontiformes) and known for their high tolerance to adverse and variable environmental conditions (Nordlie, 2006). More than half of the species within the genus are endemic to small, spring-fed water bodies and with few exceptions are distributed allopatrically in arid regions of south-western North America (Miller, 1948, 1950, 1981; Duvernell & Turner, 1998). In addition, sympatric pupfish species flocks composed of up to six morphologically distinct forms or species are known from lagunas in the Bahamas (San Salvador Island: Holtmeier, 2001) and Yucatan Peninsula (Laguna Chichancanab: Humphries & Miller, 1981; Humphries,

1984). Previous studies have documented a high potential for interspecific hybridization between numerous *Cyprinodon* species (Turner & Liu, 1977; Cokendolpher, 1980; Echelle *et al.*, 2005). Hybridization in *Cyprinodon* is most common between native and introduced species, typically involving translocated *Cyprinodon variegatus*, and generally poses major problems for the conservation of highly endemic pupfishes (Echelle & Echelle, 1997; Rosenfield & Kodric-Brown, 2003; Rosenfield *et al.*, 2004). However, natural hybridization also occurs in sympatric species assemblages on San Salvador Island and in Laguna Chichancanab (Strecker *et al.*, 1996; Strecker, 2006; Turner *et al.*, 2008), and between two species in Mexico's Cuatro Ciénegas basin (Miller, 1968; Minckley, 1969; Carson & Dowling, 2006). Although hybrids between native and introduced species have been shown to exhibit a random combination of parental traits and a greater morphological variability than parental species (Wilde & Echelle, 1997), nothing is known about the role of natural hybridization and introgression in the phenotypic diversification within the genus, despite evidence of historical introgression in several *Cyprinodon* species (Echelle *et al.*, 2005).

To study the potential effects of natural hybridization on phenotypic diversification in the genus *Cyprinodon*, this investigation focused on a system that involves natural hybridization between two pupfish species endemic to the Cuatro Ciénegas basin of the Chihuahuan desert in Mexico. Cuatro Ciénegas is a small intermontane basin with exceptional biodiversity and high levels of endemism (Ramamoorthy *et al.*, 1993). The basin is divided into an eastern and a western lobe that are partially separated by the Sierra de los Piños de San Marcos. On the northern tip of this mountain, a series of thermal springs arise from fissures along an active fault (Minckley, 1969). Springs either form isolated pools or flow into riverine systems that terminate in large evaporative lagunas or marshes. Along the transition from spring to terminal habitats, there is a steep gradient in the variability of abiotic environmental conditions (Minckley & Cole, 1968; Minckley, 1969). Thermal springs and upper reaches of riverine habitats are stable, exhibiting little variation in temperature, salinity, and pH. Peripheral habitats and terminal lagunas and marshes, in contrast, are characterized by pronounced daily and seasonal fluctuations in abiotic environmental conditions. Connection of these extremes by intermediate environments can be attenuated or abrupt. Superimposed on the abiotic environmental gradient is a less well-understood gradient in biotic conditions. In general, springs are species rich, and terminal habitats have a depauperate ichthyofauna (Minckley, 1969). This gradient is also characterized by a transition from predator-rich springs to terminal habitats that are essentially free of piscivorous fishes (Miller, 1968; Kornfield & Taylor, 1983).

At each end of the habitat continuum, one of two endemic pupfishes occurs (Miller, 1968; Carson, 2009a,b): *Cyprinodon bifasciatus* (Miller) is generally restricted to stable springs and *Cyprinodon atrorus* (Miller) to the environmentally variable terminal and peripheral habitats. The two species are not closely related and differ in their morphology, colour patterns, environmental tolerances, nuclear gene composition, and behavioural ecology (Miller, 1968; Minckley, 1969; Arnold, 1972; Echelle & Echelle, 1998; Carson *et al.*, 2008; also see Fig. 1). Aridification has led to the prolonged isolation of aquatic systems throughout the valley, which has resulted in high mitochondrial genetic differentiation between populations of both species (Carson & Dowling, 2006), as well as those of an endemic hydrobiid spring-snail (Johnson, 2005). Although *C. bifasciatus* and *C. atrorus* typically occupy opposite extremes along the environmental gradient, their ranges can overlap in environmentally intermediate habitats or at abrupt environmental junctures that often connect their preferred habitats. In these areas, hybridization is extensive, and introgressed populations are dominated by advanced backcrosses (Echelle *et al.*, 2005; Carson & Dowling, 2006). Most importantly, introgression is not only

evident in populations subject to contemporary hybridization, but persists in ones in which hybridization has apparently ceased. Whereas introgression at nuclear markers studied so far has been limited, introgression of mtDNA has been extensive, with an ancient, complete replacement of the *C. bifasciatus* mitochondrial genome by that of *C. atrorus* (Carson & Dowling, 2006).

The system of *C. bifasciatus* and *C. atrorus* provides an ideal setting for investigating the patterns of phenotypic diversification in relation to differences in environmental conditions and the incidence of introgression among populations of each species. We used a geometric morphometric approach to quantify phenotypic variation in pupfishes from different sites and relied on previously published population genetic and environmental data to address three sets of questions. (i) Is there phenotypic differentiation among different populations of each pupfish species? Based on the population genetic differentiation, geographic isolation, small population sizes, and differences in environmental conditions, it is expected that different populations exhibit different phenotype distributions. (ii) Can differences among habitat types explain phenotypic variation among populations? Because the two pupfishes and introgressed

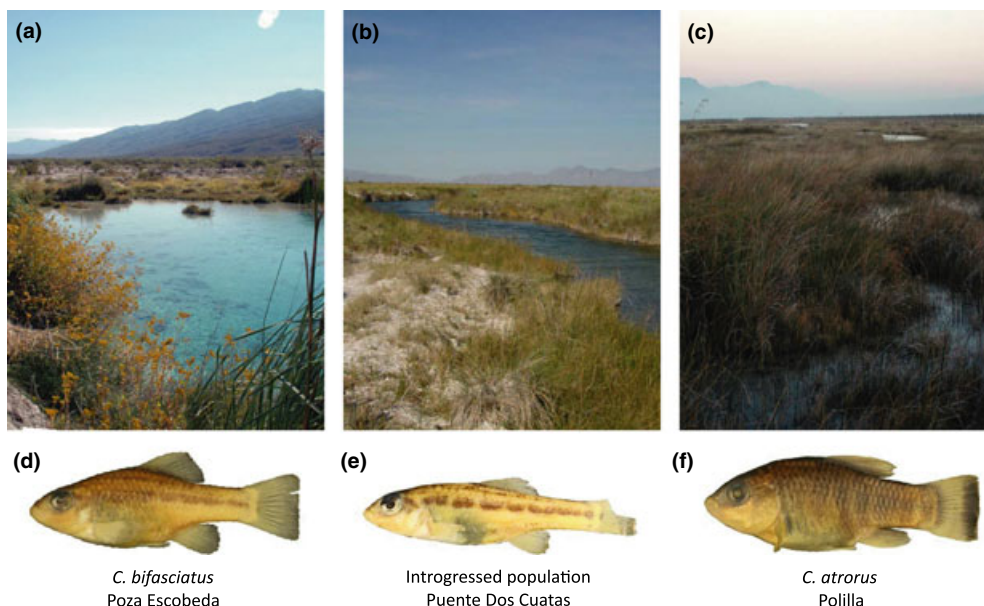


Fig. 1 Representative habitats of Cuatro Ciénegas and specimens investigated for this study. a. Poza Escobeda, a spring pool harbouring *Cyprinodon bifasciatus*. b. Puente Dos Cuatas, a site harbouring an introgressed population. c. Charcos Prietas marsh, a terminal habitat occupied by *Cyprinodon atrorus*. The lower row of pictures shows representative specimens of *C. bifasciatus* (d), of an introgressed population (e), and of *C. atrorus* (f). According to Miller (1968), *C. bifasciatus* is generally characterized by a more streamlined body shape. Males have a pale blue body with yellow eyes and usually have a thin marginal black band in the caudal fin. Females and juveniles are characterized by two eponymous, dark lateral bands on a pale brown ground colour. In contrast, *C. atrorus* are generally higher bodied. Adult males are brilliant blue with orange fins and a broad black bar on the caudal fin. Females and juveniles exhibit 5–9 faint vertical bars on the body and have a single dorsal and anal fin ocellus. Hybrids typically exhibit a combination of traits characteristic for the parental species. For example, the animal from the Puente Dos Cuatas population depicted here has lateral band characteristic for *C. bifasciatus*, but instead of being continuous, it is divided in a series of blotches reminiscent of the vertical bars typical of *C. atrorus*.

populations occur along a steep environmental gradient, phenotypic variation should be correlated with particular environmental characteristics, once genetic structure among populations and phylogenetic differences between species has been taken into account. (iii) What role does introgressive hybridization play in driving phenotypic divergence among populations that occupy different habitats? Specifically, are introgressed populations intermediate to parental species and do hybrids exhibit similar phenotypic variation as parental species? If transgressive segregation has played a significant role in phenotypic evolution of Cuatro Ciénegas *Cyprinodon*, then the phenotypic distribution of introgressed populations should lie outside that of the parental species, and introgressed populations should occupy a larger morphospace than the parental species.

Materials and methods

Collections

Pupfish were collected in 2002 throughout the Cuatro Ciénegas basin, which is located in the Chihuahuan desert (Coahuila, Mexico; see online supplementary Fig. S1 for a map). All specimens were euthanized with MS222 immediately after capture and fixed in a 10% formaldehyde solution. Table 1 summarizes the material collected and examined in the different analyses. Overall, we examined specimens from 16 sites (Table 1), which included five populations of *C. atrorus* and eleven populations of *C. bifasciatus*. Evidence for introgression stems

from previous population genetic analyses and was found in one population of *C. atrorus* and five populations of *C. bifasciatus* (Table 1, Carson & Dowling, 2006).

Assessing phenotypic variation

For geometric morphometric analysis, lateral radiographs were taken with a Hewlett-Packard (Palo Alto, CA, USA) Faxitron cabinet X-ray system. We digitized 18 landmark points on each image (see Fig. 2), using the software program tpsDig (Rohlf, 2004a). Landmarks included the tip of the upper jaw (1); the anterior tip of the supraoccipital crest (2); the posterodorsal tip of the supraoccipital crest (3); the anterior (4) and posterior (5) junction of the dorsal fin with the dorsal midline; the junction of the caudal fin with the dorsal midline (6); the last vertebra before the hypurals (7); the junction of the caudal fin with the ventral midline (8); the posterior (9) and anterior (10) junction of the anal fin with the ventral midline; the anterior junction of the pelvic fins and the ventral midline (11); the bottom of the head where the operculum breaks away from the body outline (12); the ventral tip of the maxilla (13); the centre of the first rib-bearing vertebra (14); the centre of the third vertebra with a hemal arch (15); the anterior (16) and posterior (17) tip of the parasphenoid; and the antero-dorsal tip of the gill arches (18).

Based on the coordinates of the digitized landmarks, a geometric morphometric analysis was performed (e.g. see Zelditch *et al.*, 2004 for an introduction to geometric morphometric analyses). Landmark coordinates were

Table 1 List of the collection sites, the number of individuals examined from each site in the different parts of the study, and the percent introgression at three nuclear markers, as determined by Carson & Dowling (2006). The table also provides basic information about environmental conditions at each site (based on Minckley & Cole, 1968; Minckley, 1969; Carson, personal observations).

	Sample size mtDNA	Sample size nuclear DNA	Sample size morphology	Per cent Introgression	Variation in water level	Variation in salinity	Variation in temperature	Flow
<i>Cyprinodon atrorus</i>								
Antiguos Mineros	30	30	35	0.0	High	High	High	Absent
Charcos Prietas	30	30	28	0.0	Moderate	Moderate	High	Absent
Los Gatos, spring	–	–	31	–	Low	Low	Moderate	Intermediate*
Los Gatos, marsh	42	32	31	0.0	High	High	High	Absent
Churince, marsh	30	30	29	2.8	High	High	High	Absent
<i>Cyprinodon bifasciatus</i>								
Escobedita	30	–	32	–	Low	Low	Low	Absent
Escobeda	39	39	30	0.0	Low	Low	Low	Absent
Juan Santos	40	40	30	0.0	Low	Low	Low	Absent
Mojarral Este	36	36	31	0.0	Low	Low	Low	Intermediate
Tio Candido	30	30	30	0.0	Low	Low	Low	Intermediate
Poza Churince	40	40	28	0.0	Low	Low	Low	Intermediate
Tio Julio	30	30	39	8.3	Low	Low	High	Present
Mojarral Oeste	36	36	28	0.9	Low	Low	Low	Absent
Poza de la Becerra	40	40	30	0.9	Low	Low	Low	Absent
Puente Dos Cuatas	30	60	47	1.7	Low	Low	Moderate	Present
Puente Orosco	40	40	30	0.9	Low	Low	Moderate	Present

*Fish collected in standing water, but microhabitats with flow were available in the immediate surrounding.

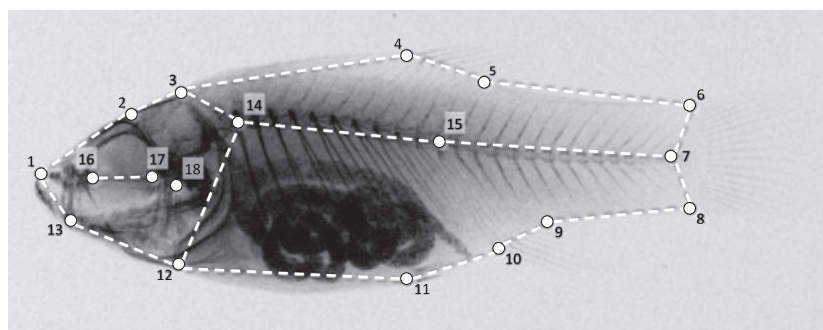


Fig. 2 Radiograph of a *Cyprinodon* highlighting the 18 landmarks used for the assessment of phenotypic variation (see main text for a detailed description of landmark positions). Landmarks are connected as in the thin-plate spline transformation grids used throughout the manuscript.

aligned using least-squares superimposition as implemented in the program tpsRelw (Rohlf, 2007) to remove effects of translation, rotation, and scale. Based on the aligned coordinates, we calculated centroid size and partial warp scores with uniform components (weight matrix) for each individual. The weight matrix was subjected to a principal component analysis based on a covariance matrix to reduce the data to true dimensionality. Null dimensions were dropped from the analysis and the remaining principal axes were retained as shape variables. Unless otherwise stated, all statistical analyses were performed using SPSS 17 (SPSS Inc.). A flowchart summarizing all analyses and data transformations performed can be found in online supplementary Fig. S2.

Phenotypic divergence among species and sites

To test for phenotypic differentiation among pupfish species and among collection sites, we used multivariate analyses of covariance (MANCOVA) to analyse body shape variation (31 principal components accounting for 99.8% of the total variation). Assumptions of multivariate normal error and homogeneity of variances and covariances were met for all analyses performed. F -values were approximated using Wilks' lambda and effect strengths by the use of partial eta squared (η_p^2). We also calculated the relative variance as the partial variance for a given term divided by the maximum partial variance value in a model. We tested for effects of 'centroid size' to control for multivariate allometry and included 'sex', 'species' (*C. atrorurus* or *C. bifasciatus*), 'introgression' (yes or no), and 'site' as independent variables to build the following nested model: intercept + centroid size + sex + species + introgression + site (species \times introgression) + sex \times centroid size + species \times centroid size + introgression \times centroid size + species \times sex + introgression \times sex + species \times sex \times centroid size + species \times introgression \times sex + introgression \times sex \times centroid size + species \times introgression \times centroid size. Shape variation along the first two principal component axes was visualized with thin-plate spline transformation grids, using tpsRegr (Rohlf, 2005). The average body shape of individuals at each site was calculated by plotting the site-specific estimated marginal means from the above MANCOVA

model against the overall mean, using tpsSpln (Rohlf, 2004b).

In addition, a discriminant function analysis (DFA) was conducted to determine the percentage of specimens that could be correctly classified to the site of origin solely based on body shape. To facilitate the DFA, we first removed the effects of sex and allometry by using the residuals of a preparatory MANCOVA. In this MANCOVA, the 31 principal components were used as dependent variables, centroid size as a 'covariate', and 'sex' as an independent variable.

Correlating phenotypes, genotypes, and environment

We tested whether phenotypic differences among populations could be predicted by species affiliation, population genetic structure, and differences in environmental conditions at the collection sites. To do this, a partial Mantel test with 10 000 randomizations, as implemented in FSTAT (Goudet, 1995; v. 2.9.3.2, <http://www2.unil.ch/popgen/softwares/fstat.htm>), was used to determine correlation of phenotypic distances among sites with genetic distances and differences in habitat conditions. As mtDNA and nuclear gene sequences were not available for the Los Gatos spring and Escobedita populations, these sites were excluded from this analysis.

To calculate pairwise phenotypic distances, the effects of 'sex' and 'centroid size' were first removed with a preparatory MANCOVA (as for the DFA above); residuals were used to conduct all further calculations. Residuals were then averaged for each population, and a matrix of Euclidean distances among all site pairs was calculated, which served as dependent variable for the partial Mantel test. Predictor matrices were based on species (same or different), pairwise genetic distances (F_{ST} values) of mitochondrial and nuclear markers, and habitat similarity. To establish pairwise F_{ST} values (calculated with FSTAT) for the populations analysed morphologically in this study, genetic data for these populations were incorporated from a previously published study (Carson & Dowling, 2006). Although haplo- and genotypes were not collected from the same individuals used for morphological analysis, the data do represent a reliable approximation of the genetic

characteristics of populations from which morphological analyses were obtained. Mitochondrial genetic distances were based on sequences of cytochrome *b* and nuclear genetic distances on sequences of creatine kinase (CK-A) intron 7, recombination activation gene (RAG-1), and triosephosphate isomerase (TPI-B) intron 4 (see Carson & Dowling, 2006 for further details). To account for broad-scale environmental differences, categorical classification of variation in water level, salinity, and temperature as well as flow regime (see Table 1) was incorporated into a principal component analysis, which was based on a correlation matrix. The first two principal component scores (explaining 96.9% of variation; online supplementary Table S1) were used to calculate environmental distances among habitat types. Shape variation along the first two principal component axes of environmental variability was visualized with thin-plate spline transformation grids, using tpsRegr (Rohlf, 2005).

Morphospace occupation and transgressive segregation

The potential for transgressive segregation in the system was assessed with a comparison of morphospace occupation of *C. atrorus*, *C. bifasciatus*, and introgressed populations at two levels. First, ANCOVA was used to compare within-site morphospace occupation among the three groups. Second, overall morphospace occupation of each group was compared to the morphospace occupation of a random group of individuals.

For this analysis, morphospace occupation is defined as the volume of a 9-dimensional convex hull enclosing all individuals of a given sample. Calculations of the convex hulls were based on the first nine principal components of body shape; as described for the other analyses previously, a preparatory MANCOVA was used to eliminate variation related to allometry and sex. The resulting residuals were then used for the calculation of the convex hull volume, using the Quickhull algorithm (Barber *et al.*, 1996; see also Cornwell *et al.*, 2006) as implemented in Matlab 7 (Mathworks Inc.). To compare within-site morphospace occupation, the convex hull volume was separately calculated for each site, and ANCOVA was used to compare among-species volumes. The number of specimens examined per site was included as a covariate in the analysis because sample sizes varied among sites, and morphospace occupation was positively correlated with sample size.

In addition, the overall morphospace occupation of *C. atrorus*, *C. bifasciatus*, and introgressed populations was calculated after lumping individuals from different sites. This was done to test whether group-specific morphospace occupation differed from that of a random assembly of individuals. As sample sizes differed among groups ($N = 125$ for *C. atrorus*; $N = 181$ for *C. bifasciatus*; $N = 203$ for introgressed populations), different randomizations

were used to reflect the respective sample sizes of each group. Random distributions of morphospace occupation were generated via 1000 iterations of an algorithm, which randomly selected – with replacement – a number of N specimens from all individuals analysed in this study and then calculated the convex hull volume of the sample. Based on the 1000 iterations, mean and standard deviation of each randomization were calculated, and the Gaussian error function was used to test whether measured group morphospace occupation differed from random expectations. These three randomizations allowed for a direct comparison of morphospace occupation between the two parentals and introgressed populations. It is expected that each parental species occupies only a subset of overall morphospace; hence, morphospace occupation should be significantly smaller than random. Introgressed populations, however, including populations that are similar to either parental species, should have a larger morphospace occupation that does not differ from a random distribution.

To test for a potential role of transgressive segregation, a fourth randomization was performed that compared morphospace occupation of introgressed populations to that of the combined parentals. To do so, random selection of specimens was limited to sites where no introgression occurred. If transgressive segregation did not play a role, morphospace occupation of introgressed populations should not differ from random (i.e. the morphospace of both parental species combined should encompass all introgressed populations). In contrast, if transgressive segregation did play a role, morphospace occupation of introgressed populations is expected to be significantly larger than that of the combined parentals.

Results

Phenotypic divergence among species and sites

Overall, 509 individuals were analysed (Table 1). Although all main effects and most interaction terms in the MANCOVA were significant (Table 2), the effects of sex and the interaction terms were generally weak ($\eta_p^2 \leq 0.19$). There were significant body shape differences between *C. atrorus* and *C. bifasciatus*, as well as between introgressed and nonintrogressed populations. Most importantly, we found shape differences among different populations of the same species (Fig. 3). This indicates substantial phenotypic variation among pupfish from different populations. Populations predominantly varied in body height, head and caudal peduncle proportions, as well as dorsal fin position along the first principal component axis (which effectively separated the two parental species), and body height, caudal peduncle proportion, and dorsal fin size along the second principal component axis (Fig. 3). *Cyprinodon atrorus* populations were generally associated with lower principal component scores on axis 1, whereas *C. bifasciatus*

Table 2 Results of the multivariate analysis of covariance (MANCOVA) that examined body shape variation of *Cyprinodon*. *F*-ratios were approximated using Wilks' lambda, effect sizes were estimated with partial Eta squared (η_p^2). Significant *P*-values and $\eta_p^2 \geq 0.2$ are given in boldface.

Effect	<i>F</i>	Hypothesis d.f.	Error d.f.	<i>P</i>	η_p^2	Relative variance
Centroid size	15.62	31.0	452.0	<0.001	0.52	1.00
Sex	1.79	31.0	452.0	0.007	0.11	0.21
Species	6.13	31.0	452.0	<0.001	0.30	0.57
Introgression	2.20	31.0	452.0	<0.001	0.13	0.25
Site (Species × Introgression)	8.85	372.0	5109.0	<0.001	0.37	0.72
Sex × Centroid size	2.98	31.0	452.0	<0.001	0.17	0.33
Species × Centroid size	3.44	31.0	452.0	<0.001	0.19	0.37
Introgression × Centroid size	2.32	31.0	452.0	<0.001	0.14	0.27
Species × Sex	1.31	31.0	452.0	0.127	0.08	0.16
Introgression × Sex	0.85	31.0	452.0	0.704	0.06	0.11
Species × Sex × Centroid size	2.26	31.0	452.0	<0.001	0.13	0.26
Species × Introgression × Sex	0.88	31.0	452.0	0.656	0.06	0.11
Introgression × Sex × Centroid size	0.88	31.0	452.0	0.663	0.06	0.11
Species × Introgression × Centroid size	2.64	31.0	452.0	<0.001	0.15	0.30

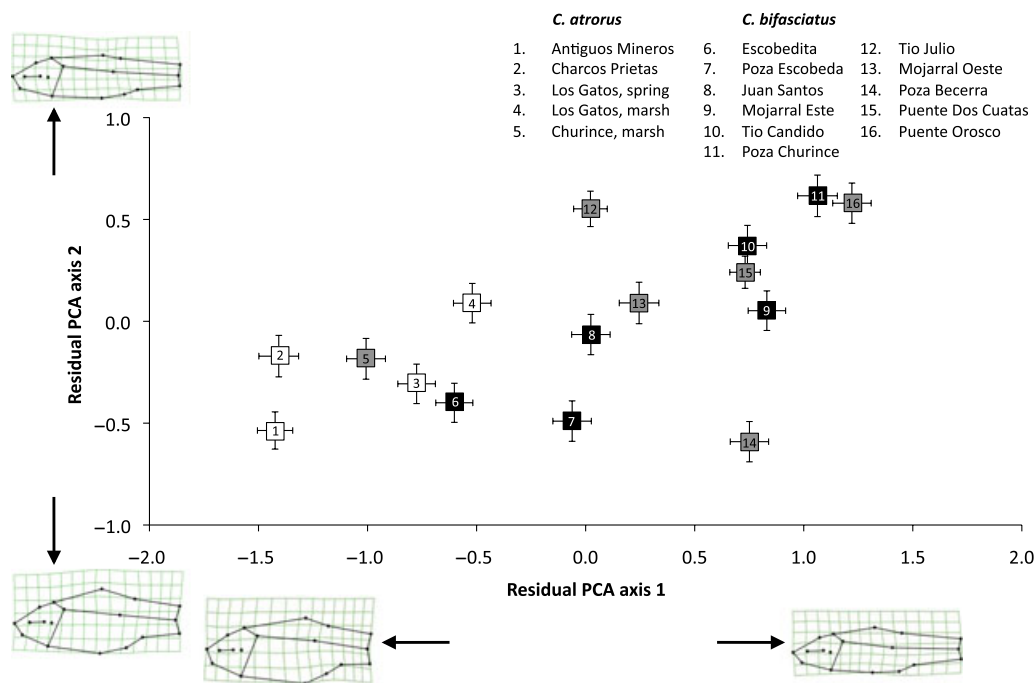


Fig. 3 Characterization of phenotypic variation in different populations of *Cyprinodon*. Depicted are mean residual principal component scores (corrected for allometric effects and sex differences, see text for explanation) and standard errors of measurement for each site. White symbols represent parental populations of *Cyprinodon atrorus*, black symbols parental populations of *Cyprinodon bifasciatus*, and grey symbols populations with evidence for introgression. Note that some introgressed populations are located within the range of one or the other parental species, whereas others lie outside of trait range exhibited by the parents. The thin-plate spline transformation grids represent shape variation along each principal component axis of shape variation.

populations were associated with higher scores on the same axis. Most importantly, introgressed populations did not exhibit intermediate phenotypes. Some introgressed populations exhibited body shapes within the range of either parental species (e.g. the Churince marsh population within *C. atrorus* and the Mojarral Oeste population within *C. bifasciatus*), but others featured

body shapes outside the range of the parental species (see also online supplementary Fig. S3).

The DFA indicated that over 87.6% of the specimens (compared to the expected 6.3% under a null hypothesis of no pattern) could be assigned to the habitat of origin solely based on geometric morphometric data (online supplementary Table S2). Along with the high effect size

of the factor 'site' in the MANCOVA, this illustrates the high phenotypic differentiation among populations and site-specific morphotypes, even within the same species.

Correlating phenotypes, genotypes, and environment

The partial Mantel test explained over 77% in pairwise phenotypic distances among sites. Whether two populations belonged to the same species was a weak predictor of phenotypic differences ($r = 0.189$, $P = 0.038$). Likewise, genetic distances based on nuclear genes did not significantly correlate with morphology (pairwise F_{ST} values of nuclear genes: $r = 0.026$, $P = 0.389$). However, genetic distances based on mitochondrial markers correlated significantly and positively with phenotypic distances (pairwise F_{ST} values of mitochondrial genes: $r = 0.397$, $P < 0.001$), accounting for about 16% of the phenotypic variation. The strongest predictor of phenotypic differences was environmental similarity among sites ($r = 0.760$, $P < 0.001$), which explained over 57% of the total variation in body shape. Principal component analysis indicated two major axes environmental variation in the system (Fig. 4, online supplementary Table S1). Along environmental axis 1, which represents a gradient from low to high environmental variability, shape variation is characterized predominantly by

changes in body height as well as head and caudal peduncle proportions; along axis 2, which represents a gradient from no flow to flow present, body shape mainly varies in terms of body height and caudal peduncle proportions.

Morphospace occupation and transgressive segregation

Within-population morphospace occupation tended to increase with sample size ($F_1 = 3.649$, $P = 0.080$, $\eta_p^2 = 0.233$), but did not differ among groups ($F_2 = 1.706$, $P = 0.223$, $\eta_p^2 = 0.221$; mean convex hull volume \pm standard deviation, *C. atrorus*: 2.30 ± 1.74 ; *C. bifasciatus*: 0.79 ± 0.40 ; introgressed populations: 2.10 ± 1.39). This result, however, needs to be interpreted with caution because the low number of sites available for the analysis constrains the statistical power. In contrast, analysis of within-species morphospace occupation indicates that introgressed populations are overall phenotypically more variable than parentals (Fig. 5 and online supplementary Fig. S4): Whereas either of the parental species only occupies a subset of total morphospace (i.e. morphospace occupation in *C. atrorus* and *C. bifasciatus* is significantly smaller than that of a random assembly of specimens; Fig. 5a and b),

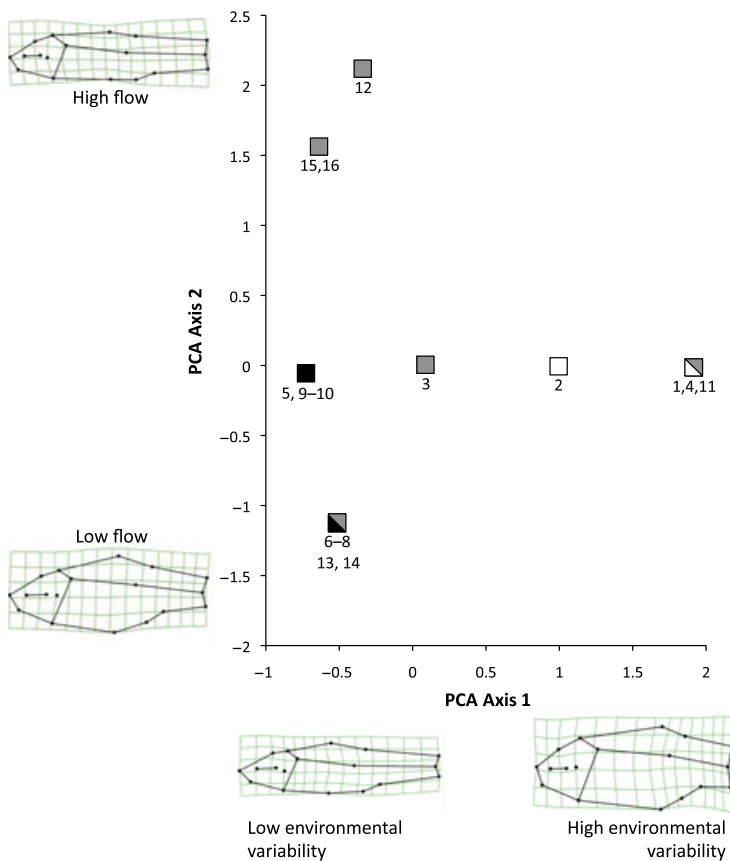


Fig. 4 Plot of the first two principal components of environmental descriptors. Along axis 1, positive scores are particularly associated with high variation in water level and salinity; along axis 2, positive scores are associated with the presence of flow. Squares represent the location of sites along the environmental gradient and are coloured white, black, and grey for *C. atrorus*, *Cyprinodon bifasciatus*, and introgressed populations, respectively. The thin-plate spline transformation grids represent shape variation along each principal component axis of environmental variation.

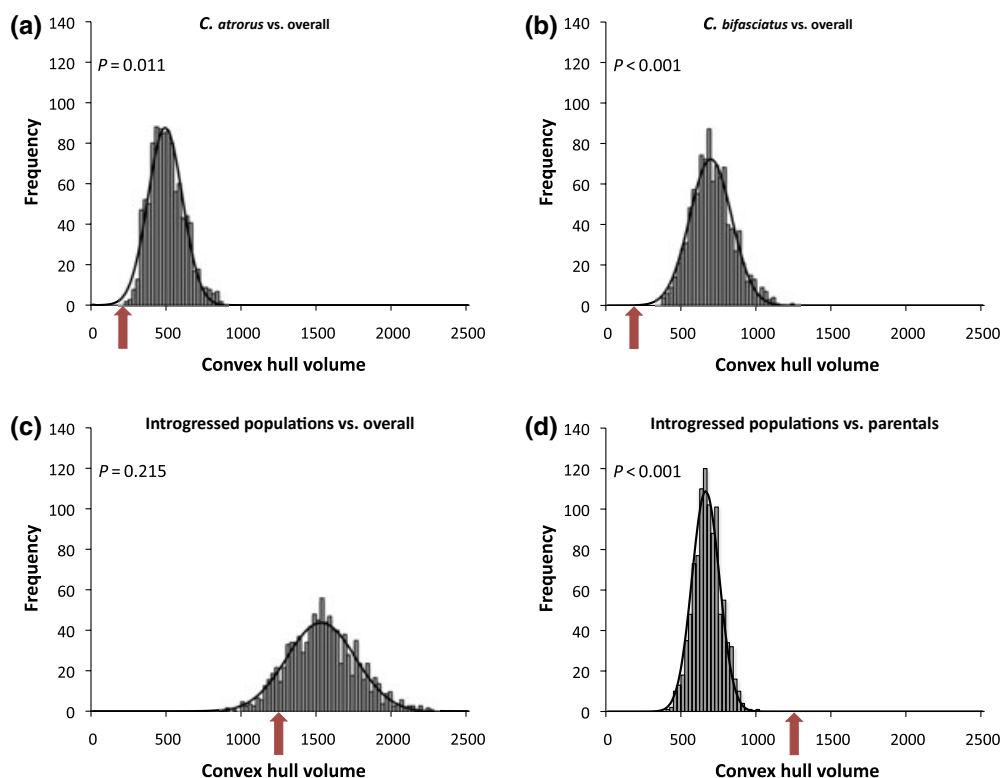


Fig. 5 Results of randomizations that depict the distribution of convex hull volumes (a) for *Cyprinodon atrorus*, based on a random selection from all specimens in the study; (b) distribution for *Cyprinodon bifasciatus*, based on a random selection from all specimens in the study; (c) distribution for introgressed populations, based on a random selection from all specimens in the study; and (d) distribution for introgressed populations, based on a random selection from specimens from parental populations. Arrows indicate the actual convex hull volume measured for a particular group. Whereas the measured convex hull volumes for the parental species are significantly lower than that of a random assembly of specimens, the introgressed populations do not differ from random expectations. However, morphospace occupation of introgressed populations is significantly higher than that of parentals alone.

morphospace occupation in introgressed populations does not differ from random expectations (Fig. 5c). This indicated that introgressed populations overall span a larger area of morphospace than each of the parentals by itself. The fourth randomization furthermore indicated that morphospace occupation of introgressed populations is significantly larger than that of the combined parentals (Fig. 5d), i.e. introgressed populations do not simply encompass the morphospace occupation by the combined parentals, but they occupy parts of morphospace that are not filled by either parental species.

Discussion

Pupfishes in the Chihuahuan desert basin of Cuatro Ciénegas provide an excellent system for the investigation of phenotypic evolution in response to differential environmental conditions and introgressive hybridization. In this study, we documented extensive phenotypic variation among isolated populations. Although we could confirm previously described morphological differences between the stout and high-bodied marsh specialist

C. atrorus and the more slender-bodied spring specialist *C. bifasciatus* (Miller, 1968), we also found significant differences along the same axes of shape variation within species. For example, *C. bifasciatus* from the Escobedita population exhibited a body shape very similar to that of *C. atrorus*. This strong phenotypic differentiation among populations even within species, in combination with the previously documented population genetic structure (Carson & Dowling, 2006), has important implications for the conservation of these threatened and highly endemic species (Carson, 2009a; b).

Although *C. atrorus* and *C. bifasciatus* generally occupy opposite extremes along a continuum of environmental variability, they hybridize extensively where their distributions overlap in intermediate habitats (Minckley, 1969; Arnold, 1972; Carson & Dowling, 2006). The introgressed populations do not exhibit simple intermediate phenotypes, but essentially span the whole range of phenotypic variation of the two parentals. More importantly, some introgressed populations (e.g. Puente Orosco, Tio Julio, and Poza Becerra) exhibit phenotypes not observed in, nor intermediate between, *C. atrorus* and

C. bifasciatus. Overall, the extensive differentiation in body shape among *C. atrorus*, *C. bifasciatus*, and introgressed populations can be explained by differences in genetic variation and local environmental conditions. Importantly, however, introgressive hybridization in this system appears to be linked to the expression of novel body shapes and the occupation of habitats with characteristics outside of those typically occupied by the parental species.

Phenotypic variation and the environment

Although genetic distance (based on pairwise F_{ST} values for a mitochondrial gene but not for nuclear genes) accounted for about 16% of the phenotypic variation, the bulk of variation was explained by local environmental conditions, as measured by the presence or absence of flow and variability of water level, salinity, and temperature. The lower explanatory power of pairwise F_{ST} values is not surprising, given the known discordant relationship between nuclear and mitochondrial gene loci in Cuatro Ciénegas pupfishes (Carson & Dowling, 2006). Genetic differentiation at nuclear loci was high between, but low within, the parental species (and most introgressed populations considered herein). Consequently, observed nuclear gene variation is largely indicative of the deep phylogenetic differences between the parental species, with the most profound differences generally coinciding with habitat differences between species and not with environmental differences among populations within species. However, the mitochondrial genome of *C. bifasciatus* was acquired through introgressive hybridization with *C. atrorus* and provides a foundation for testing whether observed phenotypic variation among populations arose through genetic drift or natural selection. Contemporary population genetic structure in both species likely arose following habitat isolation associated with ancient aridification and is largely partitioned by major hydrogeographic regions of the basin and semi-independent of species (Carson & Dowling, 2006). This pattern is consistent with the dominant role genetic drift can have in small, isolated populations. However, if genetic drift also drove phenotypic divergence among isolated populations of Cuatro Ciénegas pupfishes, more idiosyncratic patterns of phenotypic variation should have been observed (Willi *et al.*, 2006; Schluter, 2009). As such, this study adds to the extensive body of research that has documented small-scale phenotypic variation in response to divergent environmental conditions (e.g. Jimenez-Ambroz *et al.*, 2006; Hays, 2007; Langerhans *et al.*, 2007b; Manier *et al.*, 2007; Tobler *et al.*, 2008).

The uncovered relations between environmental and phenotypic variation indicate that body shape might be an important determinant of fitness along the environmental gradients. The functional significance of body shape differences remains to be tested explicitly. How-

ever, clear hypotheses can be developed from the consideration of environmental gradients in Cuatro Ciénegas and results from previously published studies on fish eco-morphology. For example, the most prominent axis of environmental variation in the system involves the gradient from very stable spring pool habitats to highly variable marshes. Along this gradient, fish vary in body height, as well as head and caudal peduncle proportions. At least two agents of selection could explain phenotypic differences that occur between gradient extremes. In springs, where piscivorous fish, such as the cichlid *Herichthys minckleyi* and the centrarchid *Micropterus salmoides*, are common (Miller, 1968; Taylor & Miller, 1980), the slender body and elongated caudal peduncle of *C. bifasciatus* would be adaptive if it promotes increased burst speeds and higher probabilities of successfully evading predator attacks (e.g. Langerhans *et al.*, 2004; Langerhans, 2009). In contrast, the adaptive significance of the stout body of *C. atrorus* in the physicochemically variable and stressful environment is less clear, but could result from selection for an optimal surface/volume ratio, as physicochemical stressors can exacerbate osmoregulatory challenges and disrupt the maintenance of homeostasis (Iwama *et al.*, 2006). Previous studies have shown that selection in response to physicochemical stressors can result in divergence of stress-related physiological pathways and be attended by morphological changes (Kobelt & Linsenmair, 1995; Langerhans *et al.*, 2007a; Tobler *et al.*, 2008). Similarly, the second axis of environmental variation predominantly considers the presence or absence of flow. The phenotypic variation along this gradient appears to represent typical phenotypic differentiation across flow regimes in fishes, and presumably involves a trade-off between steady swimming performance and manoeuvrability (Langerhans, 2008).

Two nonmutually exclusive mechanisms could explain the observed pattern of body shape differences along the environmental gradients. Phenotypic differences could be primarily driven by adaptive phenotypic plasticity, as body shape variation both in response to predation and flow has previously been documented to be inducible (Pakkasmaa & Piironen, 2001; Eklöv & Jonsson, 2007; Langerhans, 2008; Burns *et al.*, 2009). Alternatively, genetic divergence and local adaptation could drive trait divergence. The striking capacity of fish body shape to rapidly evolve differences over small spatial scales (Woods, 2007; Tobler *et al.*, 2008; Janhunen *et al.*, 2009) suggests there might have been opportunity for directional selection by local environmental conditions to produce shifts in phenotype distributions in the small, isolated pupfish populations. But although rapid differentiation in body shape has been documented in other pupfish species that were introduced into different habitats (Collyer *et al.*, 2005, 2007) or kept in artificial populations for conservation purposes (Wilcox & Martin, 2006), the underlying mechanisms of phenotypic varia-

tion have not been studied so far, and more thorough studies in the laboratory are highly warranted to resolve potential effects of phenotypic plasticity and heritable differentiation.

A role for transgressive segregation?

One of the key findings of this study was that introgressed populations of *Cyprinodon* not only occupy a wider variety of habitats than the parental species, but also exhibit greater variation in body shape, which in some cases lies outside the range of either *C. atrorus* or *C. bifasciatus*. The latter is in line with empirical confirmation that transgressive segregation is most pronounced in hybrids between more distantly related species (Stelkens & Seehausen, 2009), as would be expected for transgressive hybrids between *C. atrorus* and *C. bifasciatus*. Whereas the parental species have relatively strict habitat preferences, introgressed populations occur in habitats that are severe (Churince marsh), stable (Becerra, Mojarral Oeste), and outside the normal range of either parental species (Puente Dos Cuatas, Puente Orosco, and Tio Julio). The uniqueness of the latter habitats is exemplified by environmental variation along principal component axis 2 in Fig. 4, where these habitats exhibit relatively high-flow (unusual for *C. atrorus*) and high-temperature variability (unusual for *C. bifasciatus*).

Interestingly, there was also a discrepancy between intrapopulation and intraspecific morphospace occupation. Although introgressed populations were not more variable than parental populations within sites, they did occupy a larger across-site morphospace. This is positively correlated with the more diverse environments to which introgressed populations are exposed, and would not be expected if the morphology of introgressed pupfish were simply intermediate to parental phenotypes. These findings additionally suggest that, within the populations considered in this study, hybridization and transgressive segregation might have a limited contemporary role in shaping phenotypic variation because individual populations are characterized by relatively stabilized morphologies. This also indicates strong natural selection for the observed extra-typical phenotypes because most of these populations remain, or recently were, subject to low-level hybridization. With an important role for transgressive segregation, introgressed populations would have become isolated or nearly isolated in suitable habitats, wherein subsequent stabilizing selection condensed phenotypic variation around local optima. The apparent link between transgressive phenotypes and certain habitats suggests that environmental selection could contribute to apparent transgressive morphologies, although the influences of these two types of variation cannot be disentangled at this point. Two major scenarios could explain the origin of transgressed phenotypes, both of which might have occurred. First, transgressive phenotypes could have

been produced within the environments normally associated with the most genetically admixed pupfish populations. Under this scenario, atypical habitats would have become isolated or semi-isolated and evolutionary sorting would have winnowed the original panoply of hybrid variants down to locally adapted, transgressive phenotypes. An alternative scenario involves origination through low-level hybridization. Here, transgressive phenotypes could have originated either within environments more typical of the parental species, and subsequently colonized extraordinary habitats or become selectively favoured in the habitat of origin, or through low-level hybridization in atypical habitats that characterize the range limits of the parental species. The latter two sub-scenarios are plausible because transgressed populations display the minor levels of introgression that characterize hybrids under these two circumstances. If this did occur, ancestral transgressed genotypes could have succeeded in part because they were not subject to the magnitude of inherent evolutionary constraints known to accompany individuals of more mixed genetic heritage.

Further investigation will be required to determine the contribution of transgressive segregation to the morphological diversification of Cuatro Ciénegas pupfishes. With respect to this, three caveats merit mention. First, only three nuclear loci were surveyed and consideration of additional markers could reveal expanded occurrence of introgression. Thus, introgression could simply be coincident with the native morphological variation in Cuatro Ciénegas pupfishes or have only led to intermediate morphologies, in which case it would not be associated with transgressive segregation. However, more pervasive introgression does not necessarily negate an important role for transgressive segregation in this system. Second, Puente Dos Cuatas and Puente Orosco exhibit different morphologies, but are in relatively close proximity within a terminal riverine habitat and are unlikely to be completely isolated. It is possible that a difference in their level of contemporary hybridization with *C. atrorus* is an important component of this morphological discrepancy. Finally, the Tio Julio canal population is similar to a previously reported, stabilized hybrid swarm in the now dry La Angostura canal (Minckley, 1969). Owing to the vagaries of agricultural interests, this population has both an uncertain future and an unknown potential for hybridization with parental *C. atrorus*. However, although this population might not be in long-term morphological equilibrium or of guaranteed long-term persistence, it nonetheless appears to stabilize around a transgressive morphological norm.

Conclusions

This study documents that phenotypic variation in isolated populations of pupfishes from Cuatro Ciénegas is significantly related to environmental variation, even

after species differences are taken into account. Furthermore, transgressive segregation appears to play a role in the development of stabilized, novel phenotypes (i.e. those outside the range of parental variation) in some populations. Although delineating the relative roles of these factors will require more targeted investigation and collection of a few additional populations, the pupfishes of Cuatro Ciénegas represent an excellent system for investigating the roles environmental variability and introgression play in shaping phenotypic evolution in animals. The relatively simple environmental gradients of this system allow for explicit tests of the environmental bases of performance trade-offs. Similarly, genetic exchange is limited to two distantly related species, which reduces system-complexity and enhances the ability to discern evolutionary consequences of introgressive hybridization. Although other model systems are exceptionally valuable for similar investigations, they do have some disadvantages not present in the Cuatro Ciénegas pupfishes system, such as the confounding influences of species-richness, genetic similarity of interacting species, and/or other intrinsic properties that make it difficult to assess the adaptive function of divergent traits (e.g. in different adaptive radiations of cichlids: Seehausen *et al.*, 2003; Schliewen & Klee, 2004). Although this study has so far produced interesting correlative patterns, three major lines of investigation are required to test the hypotheses developed here. First, a more thorough understanding of the adaptive significance of divergent traits is needed. Specifically needed are rigorous tests of the performance of different phenotypes under different temperature and salinity conditions, swimming performance under different flow regimes, and susceptibility to predation. This will require consideration of other components of the phenotype, such as physiological pathways involved in coping with physiochemical stressors. Second, to understand the underlying mechanisms of phenotypic variation, laboratory common garden experiments are required. These will permit separation of the relative influences of phenotypic plasticity and heritable differentiation on trait divergence. Furthermore, crossbreeding experiments will help clarify the role of transgressive segregation in the creation of novel phenotypes. Finally, the development of additional molecular markers and implementation of more advanced genomics-based approaches will permit a more thorough understanding of the relationships between environment, introgression, and phenotype in Cuatro Ciénegas pupfishes.

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Supporting information

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

Figure S1 Map of the study region.

Figure S2 Flowchart providing an overview of all analyses and data transformations performed for this study.

Figure S3 Average body shapes of *Cyprinodon atrorus*, *Cyprinodon bifasciatus*, and introgressed populations from different sites.

Figure S4 Three dimensional convex hull volumes, representing the overall morphospace occupation, for the two parental species and introgressed populations.

Table S1 Results of the principal component analysis on environmental data.

Table S2 Canonical discriminant function analysis, using body shape data based on geometric morphometrics.

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