Relationships between spatio-temporal environmental and genetic variation reveal an important influence of exogenous selection in a pupfish hybrid zone

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Abstract

The importance of exogenous selection in a natural hybrid zone between the pupfishes *Cyprinodon atrorus* and *Cyprinodon bifasciatus* was tested via spatio-temporal analyses of environmental and genetic change over winter, spring and summer for three consecutive years. A critical influence of exogenous selection on hybrid zone regulation was demonstrated by a significant relationship between environmental (salinity and temperature) and genetic (three diagnostic nuDNA loci) variation over space and time (seasons) in the Rio Churince system, Cuatro Ciénegas, Mexico. At sites environmentally more similar to parental habitats, the genetic composition of hybrids was stable and similar to the resident parental species, whereas complex admixtures of parental and hybrid genotypic classes characterized intermediate environments, as did the greatest change in allelic and genotypic frequencies across seasons. Within hybrids across the entire Rio Churince system, seasonal changes in allelic and genotypic frequencies were consistent with results from previous reciprocal transplant experiments, which showed *C. bifasciatus* to suffer high mortality (75%) when exposed to the habitat of *C. atrorus* in winter (extreme temperature lows and variability) and summer (abrupt salinity change and extreme temperature highs and variability). Although unconfirmed, the distributional limits of *C. atrorus* and *C. atrorus*-like hybrids appear to be governed by similar constraints (predation or competition). The argument favouring evolutionary significance of hybridization in animals is bolstered by the results of this study, which links the importance of exogenous selection in a contemporary hybrid zone between *C. atrorus* and *C. bifasciatus* to previous demonstration of the long-term evolutionary significance of environmental variation and introgression on the phenotypic diversification Cuatro Ciénegas *Cyprinodon*.

Keywords: Cuatro Ciénegas basin, *Cyprinodon*, environmental variation, genotype, hybridization, nuclear introns

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Introduction

Hybrid zones have long been studied as model systems for examining the evolution of reproductive isolation and the processes of speciation. Given that hybrid zones often span divergent, complex, and temporally variable environments, it is imperative that hybrid zone studies address both spatial and temporal aspects of hybridization. However, such studies of animals have generally been confined to temporally static descriptions of hybrid zone patterns and processes. This bias is at least partially attributable to the long-standing assumption that hybrid zone dynamics are regulated primarily by endogenous selection (innate inferiority of hybrids), making the additional effort required from temporal studies less important. However, several lines of evi-
dence (Arnold 1997, 2006; Fitzpatrick & Shaffer 2004; Gee 2004; Grant et al. 2004; Culumber et al. 2011) suggest that regulation of plant and animal hybrid zones is significantly influenced by environmental (exogenous) selection, introducing the possibility that these systems can exhibit short- or long-term changes that are influenced across daily, seasonal, and more protracted ecological time frames. As such, it has become increasingly important to assess temporal as well as spatial relationships in these dynamic systems.

Several recent studies have incorporated a temporal element into analysis of hybrid zone structure and maintenance (Arntzen & Wallis 1991; Grant & Grant 1993; Gill 1997; Britch et al. 2001; Dasmahapatra et al. 2002; Gilg & Hibish 2003; Gee 2004; Grant et al. 2004; Senn et al. 2010). Results from some of these works have detected hybrid zone movement (Arntzen & Wallis 1991; Gill 1997; Britch et al. 2001; Dasmahapatra et al. 2002), superior fitness of hybrids under certain environmental regimes (Grant & Grant 1993; Grant et al. 2004; Z. W. Culumber, M. Tobler and G. G. Rosenthal unpublished data), and species/hybrid response to environmental change (Grant & Grant 1993; Gee 2004; Grant et al. 2004). Although it is likely that temporal change in many hybrid zones occurs over longer periods than covered by typical studies, several lines of evidence indicate that consideration of spatio-temporal dynamics in hybrid zones can provide insights into the dynamics of hybridization (Grant & Grant 1993; Gee 2004; Grant et al. 2004).

Investigation of the spatio-temporal dynamics of hybrid zones can potentially shed light on the relative importance of endogenous and exogenous selection. Movement of clines can be caused by either endogenous or exogenous selection (Moore 1977; Barton & Hewitt 1985; Moore and Price 1993), and ecotonal shifts can lead to similar movement between ‘tension zones’ (intrinsic hybrid inferiority) and those regulated by environmental change (Buggs 2007). However, tension zones should stabilize around a density trough, which is a localized region of low density and dispersal that might not lie in an ecotone (Barton 1979). Thus, spatial characteristics of hybrid zones maintained by endogenous selection should not track local environmental change over time, even if the hybrid zone happens to coincide with an ecotone. In contrast, cline width and placement in environmentally regulated hybrid zones should be determined by differences in hybrid fitness that relate to environmental conditions across the gradient (Moore 1977; Moore & Price 1993). An example of long-term, environmentally induced hybrid zone movement has been demonstrated for birds within the Great Plains suture zone of North America (Swenson 2006; Carling & Zuckerberg 2011). By extension, if significant selection on hybrids is driven by external environmental influences over short (e.g. seasonal) timescales, changes in the abundance, distribution, and genetic composition of hybrids should also respond to such environmental variability. For a given hybrid zone, its distributional characteristics and temporal variation will depend on the relative contributions of endogenous and exogenous selection to system regulation.

Hybridization between two pupfish species, Cyprinodon atrorus and Cyprinodon bifasciatus (Miller 1968), endemic to the Cuatro Ciénegas basin, Mexico, presents an excellent system to conduct spatio-temporal analyses of the relationship between environmental and genetic variation in hybrid zones. These species are short-lived and are known to have multiple cohorts per year (Arnold 1972). This increases opportunity for response to short-term changes in the environment and thus provides an opportunity to investigate patterns of genetic variation across generations and seasons within a hybrid zone. In addition, these species occupy different habitat extremes in a desert spring ecosystem, with C. atrorus found in environmentally variable habitats of the basin floor (primarily marshes, playa lakes, riverine edges and ephemeral pools) and C. bifasciatus in physicochemically moderate to constant thermal springs and their environmentally buffered riverine outflows (Minckley 1969; Carson 2005, 2009a,b).

Reciprocal transplant experiments with C. atrorus and C. bifasciatus (Carson et al. 2008) indicated that C. bifasciatus is aberrant within the genus in that it suffers high mortality when exposed to severe (i.e. C. atrorus) habitats; in contrast, C. atrorus exhibits low mortality in benign (i.e. C. bifasciatus) habitats. It is thought that C. atrorus, typical of species in the genus, is highly susceptible to predation and is therefore prevented from occupying habitats of C. bifasciatus, which harbour a diverse array of native, predatory fish species (Minckley 1969). Cyprinodon bifasciatus exhibits morphological and behavioural adaptations (e.g. fusiform body, schooling/aggregating) that likely make it less susceptible to predation; in contrast, C. atrorus is a squat-bodied fish that does not possess the sophisticated predator-evasion responses of C. bifasciatus (Minckley 1969; Arnold 1972; Tobler & Carson 2010). Carson et al. (2008) suggested that other factors, such as competitive exclusion, could also prevent C. atrorus from becoming established within habitats dominated by C. bifasciatus (see Echelle et al. 1972).

Despite these differences, C. atrorus and C. bifasciatus exhibit range overlap along the environmental gradient that separates their typical habitats, and within these areas of sympatry hybridization can be extensive (Miller 1968; Minckley 1969). Analysis of hybridization on a large spatial-scale (basin-wide) indicated a strong asso-
ciation between introgression and environmental characteristics (Tobler & Carson 2010) and suggested that hybridization between these species could be governed, at least in part, by exogenous selection. How exogenous selection is manifested over time, though, remains unknown. *Cyprinodon bifasciatus* has a tendency to extend its range downstream in summer, as conditions become more spring like, and contract its range in winter, when downstream environments become too cold (Miller 1968; Minckley 1969). This cyclical change in distribution suggests the relationship between genetic and environmental variation in hybrid zones between these species might also respond to seasonal environmental change.

To test for the influence of exogenous selection in hybrid zones between *C. atrorus* and *C. bifasciatus*, spatio-temporal sampling of environmental and genetic variation across the Rio Churince system was conducted in winter, spring and summer over three consecutive years. Extensive natural hybridization between *C. atrorus* and *C. bifasciatus* occurs in association with the Rio Churince (Fig. 1). This system is relatively small (approximately 3.0 km long) and thus easily tractable for hybrid zone studies. Environmental variables included specific conductance and temperature, and genetic variation was assessed at three nuclear DNA loci for which allelic variation is diagnostic between these species (Carson & Dowling 2006). Specifically, we addressed the following three questions about the importance of exogenous selection in the Rio Churince hybrid zone: (i) is there spatial and temporal variation in the physicochemical environment and genetic composition of *Cyprinodon*? (ii) How does environmental variation relate to genetic composition of *Cyprinodon* across sites? (iii) Over the entire Churince system, are there seasonal patterns of variation in the frequency of hybrids and allele frequencies within hybrids? For physicochemical-based exogenous selection to occur, spatial and temporal (either or both) change in environmental and genetic variation is required. Within this context, spatial variation in the genetic composition of subpopulations should reflect local environmental conditions, and the spatial distribution of genetic variation within hybrids should track seasonal changes in the environment. Thus, more severe environments should be dominated by the *C. atrorus* genotype and alleles.

![Fig. 1 Map of the Rio Churince system and collection sites (Chu01–17). Poza Churince (headspring; Chu01) feeds the Rio Churince (Chu02–03, 11 and 13–15), which terminates in Laguna Grande (Chu16–17). The Rio Churince and a large marsh system (Chu06) feed Laguna Intermedia (Chu04). Peripheral habitats are Chu05, 07–10 and 12; Chu11 contains riverine and peripheral aspects. The three relatively distinct physiographic segments of the Rio Churince system are as follows: (i) the upper reach (Chu01–03) is characterized by a 1–3-m-deep source spring, its immediate, meandering outflow (approximately 1 m deep and wide) and relatively poor development of peripheral habitat; (ii) the middle reach (Chu04–011) is comprised of the large, shallow Laguna Intermedia, the broad (1–2 m) and shallow (<1 m) Rio Churince, and extensive peripheral and marsh habitats; and (iii) the lower reach (Chu12–17) consists of a severely restricted, shallow channel that is interrupted by pools, is lined by small but numerous marsh and peripheral habitats and terminates in the evaporative Laguna Grande.](image-url)
specific to this species and more benign environments should be dominated by *C. bifasciatus* and alleles specific to this species; in contrast, physicochemically intermediate habitats should have a corresponding intermediacy of allele and genotype frequencies, which would indicate a large proportion of hybrids. Finally, within-site characteristics of genetic variation in hybrids should be labile and show predictable response to seasonal environmental change.

**Materials and methods**

**Study system**

The Rio Churince system (Fig. 1) was selected as a model for the examination of hybridization between *Cyprinodon atrorus* and *Cyprinodon bifasciatus*, as it contains the only extensive, contemporary hybrid zone known to occur in the Cuatro Ciénegas basin. Carson (2005) characterized Rio Churince system habitats, which range from physicochemically constant and benign (Poza Churince and upper reaches of the Rio Churince) to environmentally severe (Laguna Grande, marshes, and some peripheral habitats). Intermediate environments typify middle and lower reaches of the Rio Churince and some of its peripheral habitats, with the characterization of the latter depending on season and/or locality. Transition from stable to severe habitats occurs across a major linear gradient (Poza Churince to Laguna Grande) that is also intersected perpendicularly by multiple, shorter gradients that connect harsh peripheral or marsh habitats to the Rio Churince. The most severe habitats are dominated by *C. atrorus*, the most stable ones by *C. bifasciatus* and intermediate ones by hybrids and one or both parental species (Carson 2005). Thus, habitats within the Rio Churince system consist of a spatially complex partitioning of parental species, hybrids and environmental variation, with the hybrid zone containing elements of both linear and mosaic structure.

**Quantifying environmental variation**

Specific conductance (a proxy for salinity) and temperature variation were measured over three consecutive years to characterize spatial and temporal environmental variation in the Rio Churince system. In year 1, 14 sites (Chu 1, 2, 4–6, 9–17; Fig. 1) were sampled for two of the sampling dates (December 2000 and March 2001); all seventeen sites (Chu 1–17; Fig. 1) were sampled in the third sample date from year 1 (August 2001) and in all sample dates for years 2 (January, March and August 2002) and 3 (January, March and August 2003). A YSI 85 (YSI Inc.) was used to measure specific conductance, and iButtons (Maxim Integrated) were used to record temperature variation. iButtons were positioned approximately 15–25 cm deep in each habitat to maintain consistency across sites and to capture temperature profiles that would have been experienced by resident *Cyprinodon* during each survey period. Temperature was recorded every hour for 1–3 weeks, depending on the length of the sampling trip. Temperature sensors were not used for the December 2000 collection.

**Specimen procurement and characterization of genetic variation**

In conjunction with measurement of environmental variation, samples of *C. atrorus*, *C. bifasciatus* and their hybrids were collected at the sites and dates listed under the Quantifying environmental variation section above. All specimens were collected with a seine or dip-net, with individuals selected haphazardly to reduce collection bias. In accordance with permit limitations, 30 specimens were collected for each site and date. Specimens were preserved in 95% ethanol, and the phenol–chloroform protocol of Tibbets & Dowling (1996) was used to extract DNA from muscle tissue of each sample.

A previous survey (Carson & Dowling 2006) of the nuclear genes creatine kinase-A (CK-A7, intron 7), recombination activating gene (RAG-1, partial exon 1) and triosephosphate isomerase B (TPI-B4, intron 4) found that alleles at these loci are diagnostically different between *C. atrorus* and *C. bifasciatus* and therefore suitable for investigation of hybridization between these species. Sequence variation at CK-A7, RAG-1 and TPI-B4 in *Cyprinodon* from the Rio Churince system was characterized through analysis of single-stranded conformational polymorphisms (Glavac & Dean 1993; Takeda et al. 1995), using the methods of Carson & Dowling (2006). GenBank accession numbers for DNA sequences of the diagnostic nuclear gene markers (CK-A7, RAG-1 and TPI-B4) are EU086365–EU086370. For each of the loci surveyed in the Rio Churince system, only two alleles were found, which correspond as follows to those of Carson & Dowling (2006): cA1, rA1 and tA1 for alleles specific to *C. atrorus* and cB1, rB1 and tB1 for alleles specific to *C. bifasciatus* at the CK-A7, RAG-1 and TPI-B4 loci, respectively.

**Spatial and temporal variation in environmental conditions**

To analyse variation in environmental characteristics, mean specific conductivity, mean temperature and standard deviation of temperature, as well as temperature
Spatial and temporal variation in genetic composition

To test for spatial and temporal variation in allele frequencies, multilocus genotype information was reduced via principal component analysis (see Cavalli-Sforza & Feldman 2003; Patterson et al. 2006; % variance explained: 76.9; Factor loads: CK = 0.863, CK-2 = 0.890, RAG = 0.891, RAG-2 = 0.875, TPI = 0.883, TPI-2 = 0.861). Individual principal component scores thus reflect the relative contribution of *C. atrorus* and *C. bifasciatus* alleles to an individual’s genetic composition, where negative scores correspond to *C. atrorus*-like genotypes and positive scores to *C. bifasciatus*-like genotypes. The individual scores were averaged for each site and sampling period, describing the average composition of populations at each time. Spatial and temporal variation in the average composition was analysed using a fully factorial repeated measures linear mixed model with average genotype composition (principal component (PC) score) as the dependent variable and site, season and year as random factors.

Relating environmental variation to hybridization

To relate population-level allele frequency to (spatial) environmental variation, the overall mean allele frequency score from the PCA was calculated for each population (across all sampling periods) and used as a dependent variable in a multiple regression. Overall means and coefficients of variation of both PC axes of environmental variation were used as predictor variables. The multiple regression was run with a backwards elimination approach; however, other methods (full model, forward approach) yielded identical results. A quadratic regression between mean genotype frequency and the coefficient of variation of genotype frequency (LN-transformed) was used to test whether variability in allele frequencies was even across sites or highest at sites where hybridization was pervasive.

Seasonal variation

To better understand the effects of seasonal variation on hybrid zone dynamics, two complementary analyses addressed the overall composition of the Churince pupfishes (i.e. fish from different sites were pooled for these analyses). First, to test whether there were any seasonal effects on the frequency of hybrids in the system, individuals were classified as parental (*C. atrorus* or *C. bifasciatus*) or hybrids. A G-test was used to examine whether there were significant deviations from expected frequencies across time. To visualize these deviations, relative deviation for each season and year was first calculated and then the values across years were averaged. Second, to test whether there were any seasonal effects on the genetic composition of hybrids in the entire Churince system, fish from all sites were again pooled, but individuals that possessed a parental genotype were excluded. Genotype composition (in the form of PC scores, as calculated above) was then analysed using GLM with season and year as random factors.

Results

Spatial and temporal variation in environmental conditions

Principal component analysis identified two axes that explained the majority of environmental variation (Table S1 and Fig. S1, Supporting information). Mixed models found significant variation along both axes (Table 1) and predominantly identified differences among sites and site-specific seasonal variation (the site × year interaction term was also significant for axis PC2). In essence, specific conductance was stable and similar across most sites, with a slight and gradual linear increase from the springhead to terminal Laguna (Fig. S2, Supporting information). In contrast, there was substantial seasonal temperature variation within and among sites. Uppermost sites (CHU01-02) were characterized by near constant temperatures year-round and average temperatures across the gradient attenuated from winter to summer to become approximately the same across the entire system (Fig. S2, Supporting information). However, terminal (Chu16-17), marsh (Chu06) and peripheral (Chu05, 07-10 and 12) habitats exhibited the greatest absolute temperature variability (as measured by range and standard deviation) both within seasons and across seasons (Fig. S2, Supporting information). These latter sites also experienced substantial declines in water level in summer, but all other sites (Chu01-04, 11, 13–15) showed minor seasonal differences in inundated surface area. Detailed description of spatio-temporal environmental variation is provided in Appendix S1 (Supporting information).

Summary of genetic variation

Across all sites and years, a total of 4161 *Cyprinodon* was genotyped for three nuclear loci, with 40.6% (1690)
The interactions ‘site × season × year’ and ‘season × year’ as well as the factor ‘year’ were redundant parameters and thus omitted from the final model. Environmental variation (PC 1) and (C) environmental variation along PC 2. Significant P-values are indicated in bold.

*The interactions ‘site × season × year’ and ‘season × year’ as well as the factor ‘year’ were redundant parameters and thus omitted from the final model.

†The interaction ‘site × season × year’ and the factor ‘year’ were redundant parameters and thus omitted from the final model.

of these represented by genotypically ‘pure’ Cyprinodon atrorus and 26.9% (1121) ‘pure’ Cyprinodon bifasciatus (see Fig. 2). Hybrids were very common in the system, comprising approximately 32.5% (1350) of all individuals. Cyprinodon atrorus-like hybrids were 13.5% (562) of all fish and 41.6% of hybrids; C. bifasciatus-like hybrids made up 11.5% (476) of all fish and 35.3% of hybrids. In contrast, intermediate hybrids (including putative F1 individuals) were not as common, representing 7.5% (312) of all specimens and 23.1% of hybrids. Counts of parental, C. atrorus-, intermediate and putative hybrids were 13.5% (562) of all fish and 35.3% of hybrids; however, F1 individuals or backcrosses. Average levels of introgression across loci were low (1–3%) at parental C. atrorus sites (Chu06, 16,17), but introgression at parental C. bifasciatus sites (Chu01–03) was limited to one collection (Chu03 in Spring 2002) that contained an individual that possessed a C. bifasciatus-like hybrid genotype (Table S3, Supporting information).

Spatial and temporal variation in genetic composition

The mixed model identified significant variation in average genotype scores across sites (Table 1, Fig. 3). Furthermore, there was seasonal variation in some populations (significant season by site interaction). Although temporal variation in allele frequency variation was minor at sites where parental genotypes dominated, intermediate habitats with pervasive hybridization exhibited pronounced temporal variation in average allele frequencies (also see below). There was also a marginal interaction effect between site and year, which indicates that fluctuation in the frequency of hybridization could have varied annually.

Relating environmental variation to hybridization

Environmental variables significantly explained variation in mean genotype frequencies across populations (model \(R^2 = 0.711, F_{1,16} = 36.895, P < 0.001\)). Mean score along environmental axis 1 was the only variable retained in the model (Fig. 4A; \(B = 1.122 \pm 0.185, t = 6.074, P < 0.001; \) Table 1); parental species predominately occurred at the extremes along environmental axis 1, and hybridization was most common in intermediate habitats.

Variability in gene frequency within sites was significantly related to the mean genotype frequency (Fig. 4B; quadratic regression: \(R^2 = 0.798, F_{2,14} = 27.686, P < 0.001\)). This indicates that population composition was relatively stable at either end of the spectrum along environmental variation axis 1 (i.e. where the parental species predominate). In contrast, hybridization was most common and genotype frequencies were most variable over time within intermediate habitats.

Seasonal variation

Frequencies of parental species and hybrids varied significantly across seasons (\(G_{16} = 103.74, P < 0.001; \) Fig. 5A). In the winter, hybrids were significantly over-represented in the Churince system, while both parental species were underrepresented. In spring, deviations from random expectations were not significant, but during summer, parental species dominated significantly more.
over hybrids. Thus, the frequency of hybrids exhibited clear seasonal changes.

Seasonal effects on the allele frequency within hybrids in the Churince system were also significant ($F_{2,4.05} = 21.705, P = 0.007$; Fig. 5B). During winter, allelic composition of hybrids was biased towards C. bifasciatus. In contrast, during spring and summer, there was a significant bias towards C. atrorus alleles. There was no significant effect of year ($F_{2.4.09} = 1.447, P = 0.335$) or the season by year interaction ($F_{4.1341} = 0.916, P = 0.454$).

**Discussion**

In the study of animal hybrid zones, a critical but longstanding uncertainty is centred on the importance of exogenous selection (Barton & Hewitt 1985; Arnold 1997, 2006). Consequently, it also remains unsettled whether hybridization has been of broad evolutionary significance in animals (Barton & Hewitt 1985; Arnold 1997, 2006). Resolution of these questions has been hampered, in part, by a dearth of temporal investigations that permit detection of important seasonal and annual variation in hybrid zones. To test for the influence of exogenous selection in an animal hybrid zone, the present study investigated relationships between spatio-temporal change in environmental and genetic variation in a hybrid zone between the pupfishes Cyprinodon atrorus and Cyprinodon bifasciatus. Results from this study enhance understanding of the contemporary and historical influences of hybridization in Cuatro Ciénegas Cyprinodon and, more broadly, provide insights relative to the evolutionary significance of introgressive hybridization in animals.

**Relationships between spatio-temporal characteristics of environmental and genetic variation**

Genetic composition of Cyprinodon in the Rio Churince system was not only variable in space and time, but environmental variation explained this change. Importantly, hybrid genotypes were abundant throughout the duration of the study and most hybrids were represented by backcrosses or other advanced genotypic mixtures (approximately 42% ‘C. atrorus-like’ and approximately 35% ‘C. bifasciatus-like’). Sites dominated by parental genotypes were the most environmentally benign (upper Rio Churince, C. bifasciatus) or severe...
Fig. 3 Variation in mean genotype composition of 17 populations across seasons and years. Genotype composition was determined based on PC scores of multilocus genotypes for each individual (see text). Positive values correspond to an excess of *C. bifasciatus* alleles and negative values to an excess of *Cyprinodon atrorus* alleles.

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Fig. 4 (A) Relation between environmental axis 1 and mean genotype frequency at each site. Both genotype composition and environmental values are based on PC scores. Positive values along the genotype axis correspond to an excess of *Cyprinodon bifasciatus* alleles and negative values to an excess of *Cyprinodon atrorus* alleles. For axis loadings for the environmental variables, see Table S1 (Supporting information). (B) Relation between mean genotype composition (PC score) and the coefficient of variation of genotype composition.

Fig. 5 (A) Seasonal variation in the frequency of parental and hybrid individuals. Depicted are mean relative deviations (±SE) between observed and expected frequencies based on a G-test. Positive values indicate a higher frequency than expected and negative values a lower frequency than expected. (B) Variation in mean genotype composition (±SE) of hybrids in the entire Churince system for each season. Increasing positive scores are associated with greater allelic contribution from *Cyprinodon bifasciatus*, and scores approaching zero reflect increased allelic weighing towards *Cyprinodon atrorus*. (Peripheral and terminal sites, *C. atrorus*) and, not surprisingly, subpopulations at these sites showed the least variation in genotype and allele frequencies over time. However, environmentally intermediate sites were host to pervasive hybridization, and subpopulations within these sites showed pronounced variation in genetic composition over seasons. Thus, the Rio Churince hybrid zone is not characterized by a gradual and stable transition from one parental form to another but instead showed significant spatial structure that is related to environmental change. Furthermore, subpopulations that exhibited the highest frequency of hybrid genotypes (intermediate environments) also were most prone to temporal changes in genetic composition over seasons. Close correlation between spatio-temporal environmental and genetic change is not easily explained by a strict adherence to a model of regulation by endogenous selection but is expected if system dynamics are driven by exogenous selection. Further delineation of genetic variation among hybrids reveals additional evidence that exogenous selection is likely to be important. For example, hybrids in the more environmentally severe and benign sites were genetically similar to *C. atrorus* and *C. bifasciatus*, respectively. Among hybrids, exclusive occurrence of *C. atrorus*-like hybrids in habitats dominated by parental *C. atrorus* is not explained by an inability of other hybrids to disperse to these sites, as other hybrid genotypic classes occur in appreciable frequencies close to severe habitats and intervening physical barriers are not present to limit movement between these sites. Thus, hybrid classes genetically dissimilar to *C. atrorus* are likely prevented from entering these harsh environments or face severe negative selection if they do immigrate. Such isolation via selection against immigrants (Nosil *et al.* 2005) has been documented in other aquatic systems that exhibit pronounced variation in environmental characteristics over small spatial scales (Tobler...
et al. 2009). Similarly, absence of *C. atrorus*-like hybrids from *Cyprinodon bifasciatus* habitat is also consistent with selection imposed by exogenous factors, as *C. atrorus* subpopulations occur adjacent to and have variable but direct contact with the upper Rio Churince. *Cyprinodon atrorus* is thought to be typical of most pupfish species in suffering a high susceptibility to predation by piscivorous fishes, which presumably reflects a long evolutionary history of limited co-occurrence with other fishes, especially large predatory ones (see Tobler & Carson 2010 for a discussion). This is supported by the high survivorship (100%) of *C. atrorus* in *C. bifasciatus* habitat in summer and winter reciprocal transplant experiments that excluded predatory fishes (Carson et al. 2008). High predation or other exclusionary factors appear to extend to *C. atrorus*-like hybrids, but additional experiments will be required to determine the relative predation susceptibility of *C. atrorus*, *C. bifasciatus* and their hybrids.

Seasonal variation and exogenous selection

Seasonal variation in the frequencies of parental species and hybrids was significantly related to variation in environmental conditions. Hybrids predominated over both parental species in winter, but by spring the frequencies of parental species did not deviate from random expectation and by summer parental species were dominant. The frequency of hybrid genotypes across the entire system showed strong and predictable response to seasonal environmental change. Importantly, this change is not explained by dispersal, as comparison encompasses the entire Rio Churince system. This change is also not explained by simple increases and decreases in abundance of parental species. Despite evidence that the frequency of hybridization could have varied annually among sites, there was no significant change in allele frequencies of hybrids either within seasons or within seasons among years. Thus, selection on hybrid genotypes varied among seasons but remained predictable over time.

A significant influence of exogenous selection is, in fact, apparent from the pattern of temporal allele frequency change within hybrids, with the clearest influence of exogenous selection having occurred between winter and spring. Across this period, allelic frequencies in hybrids shifted from a bias towards alleles specific to *C. bifasciatus* in winter to bias towards alleles specific to *C. atrorus* in spring. Reproduction rates are low in both species during this period and decline gradually from summer to the following spring in *C. atrorus* (Arnold 1972). Changes in reproductive activity of the parental species are unlikely to account for observed winter to spring changes in the genetic composition of hybrids. Ripe ova are reduced in females of both species in winter, and the size of *C. atrorus* young of the year through mid-spring (end of April) is <15 mm (Arnold 1972) and smaller than the minimum collection size from this study. The winter to spring shift in genetic characteristics of hybrids is, however, consistent with exogenous selection; only adults and near-mature juveniles were sampled, and the selective response over this period could stem from an acute and/or short but cumulative effect (i.e. time lag). Results from a reciprocal transplant experiment in this system (Carson et al. 2008) suggest the selective mechanism could be related to temperature tolerance differences among hybrids. Specifically, the winter experiment from that study revealed *C. bifasciatus* to exhibit high mortality (75%) in a habitat typical of *C. atrorus* (i.e. cold and variable temperature). If temperature tolerance differences between parental species extend to their most genetically similar hybrid counterparts, observed winter to spring reductions in alleles specific to *C. bifasciatus* likely resulted from negative response to acute or cumulative cold stress, as despite desert conditions, the basin regularly experiences cold conditions in winter. This possibility is also more generally supported by a recent niche modelling study of a North American suture zone (Swenson 2006), wherein hybrid zones in four species pairs of birds revealed that temperature holds the suture zone together and could have been a primary influence on Pleistocene niche differentiation of each species pair (but see the study by Emlen et al. 1975 for counter arguments for one of these pairs).

The frequency-bias towards alleles specific to *C. atrorus* advanced further by summer. This is consistent with the summer reciprocal transplant experiment of Carson et al. (2008), which showed *C. bifasciatus* to exhibit high mortality (75%) in an environment subject to high mean and variance in temperature, as well as severe changes in salinity over a short period. Although, owing to necessities of experimental simplification, hybrids were not used in that experiment, results from the present study suggest the same exogenous factors that favour *C. atrorus* in highly variable environments also have negative effects on the fitness *C. bifasciatus*-like hybrids and potentially positive effects on *C. atrorus*-like hybrids. However, change in genetic composition of hybrids between spring and summer is confounded by potential differences between the life-history characteristics of the parental species. Specifically, although *C. atrorus* can breed from January through November (Deacon & Minckley 1974), reproduction in this species occurs at a low rate in colder months and peaks in spring (Arnold 1972). In contrast, *C. bifasciatus* lacks a distinct breeding season (Arnold 1972). Abundance of
C. atrorus can be very high by summer; thus, it is possible that increases through summer in the frequency of alleles specific to C. atrorus are related, at least in part, to numeric advantage in reproduction by this species between spring and summer. However, seasonal return to a C. bifasciatus-like bias of hybrids by winter remains unexplained. If reproductive activity favours C. atrorus and C. atrorus-like offspring in the mid-to-late reproductive cycle, these genotypes should also dominate in early-to-mid winter collections, but this was not observed. This change could have underpinnings of exogenous selection if, for example, the life cycle of C. atrorus-like hybrids is similar to that of C. atrorus (i.e. an important driver of the summer to winter, within-hybrid decline in frequency of alleles specific to C. atrorus). Further research is needed to resolve these uncertainties.

Relationships between hybridization, exogenous selection and evolutionary diversification

With the exception of the Rio Churince system, known cases of contemporary hybridization between C. atrorus and C. bifasciatus are limited to numerous but small-scale, sporadic or ephemeral situations (Carson 2005). However, it is certain that in the distant past hybridization between these species was common and geographically widespread, as ancient hybridization led to range-wide, complete replacement of the C. bifasciatus mitochondrial genome by that of C. atrorus (Echelle et al. 2005; Carson & Dowling 2006). It is unknown whether replacement occurred through neutral processes or resulted from positive selection for the C. atrorus mtDNA genome. Although a role for positive selection cannot be dismissed, this possibility probably cannot be tested because the mitochondrial genome of C. bifasciatus is apparently extinct. However, several characteristics of the Cuatro Ciénegas Cyprinodon system are consistent with, though not necessarily exclusive to, a nonadaptive basis of genome replacement, including (i) the geographic distribution of C. atrorus surrounds all populations of C. bifasciatus, (ii) the placement of hybrid zones is centred close to the range centre of C. atrorus, and (iii) C. atrorus females have a reproductive advantage in contact zones (Ludlow 2000). Regardless of how replacement occurred, acquisition of the C. atrorus mitochondrial genome by C. bifasciatus has been of evolutionarily significance.

In contrast to mitochondrial genome replacement, nuclear DNA introgression appears low or absent in semi-isolated to fully isolated populations of both species (Carson & Dowling 2006). Nuclear gene introgression is also relatively restricted, though asymmetric, in the Churince system, where levels are low but persistent in C. atrorus and negligible in C. bifasciatus. Asymmetric introgression in this case probably stems in part from the location of the Churince hybrid zone, which is centred closer to habitats typical of C. atrorus than to those most characteristic of C. bifasciatus. However, the combination of extensive hybridization (past and present), low levels of introgression and well-maintained species differences in Cuatro Ciénegas Cyprinodon could be indicative of the adaptive significance of hybrid zones between C. atrorus and C. bifasciatus. This possibility is supported by a cottonwood hybrid zone study by Martinsen et al. (2001), which revealed not all genomic regions are equally porous to introgression. This finding led the authors to argue that hybrid zones effectively ‘filter’ inter-specific gene flow and that a strong filter may not only prevent fusion of parental species but can also make introgression adaptive by facilitating transfer of advantageous variation but arresting that of deleterious genes. Thus, the relatively low levels of nuclear DNA introgression observed in the Churince hybrid zone not only reflect strong reproductive isolation but also may be indicative with significant transfer of adaptive variation between species. The disparity in degrees of introgression between the mitochondrial and nuclear genomes (for the few markers studied to date) in the Churince hybrid zone demonstrates that significant transfer of genetic variation can occur despite a strong selection gradient and suggests that the transfer of adaptive variants in particular is also possible.

Historic nuclear DNA gene exchange also occurred between C. atrorus and C. bifasciatus (Carson & Dowling 2006) and has apparently led to permanent acquisition of adaptive variance in both species, as a basin-wide study by Tobler & Carson (2010) found that environmental variation and introgression at nuclear DNA loci significantly explained phenotypic variation among semi- to long-isolated populations of Cuatro Ciénegas Cyprinodon. The basic contemporary character of environmental gradients and habitat preferences of these species would almost certainly have been present throughout relevant evolutionary history; therefore, it is reasonable to assume that exogenous selection would have operated similarly throughout the long history of genetic interaction between these species. Based on mtDNA sequence divergences among Cyprinodon populations in the basin (<1%; Carson & Dowling 2006), many of the major habitats that characterize the Cuatro Ciénegas basin today most likely became semi-isolated to fully isolated as a consequence of Quaternary climate change, which had similar effects on numerous species in this region and throughout the world (Heviit 2000, 2004). Because mtDNA replacement is ancient and nuclear gene introgression is present in semi- and long-isolated populations, long-term population
fragmentation in Cuatro Ciénegas Cyprinodon would have allowed introgressed isolates to evolve with heterospecific-derived genetic variation at their adaptive disposal.

The importance of exogenous selection in the Churince hybrid zone and the adaptive significance of historical introgression to the phenotypic diversification of C. atrorus and C. bifasciatus bolster the increasingly persuasive argument that evolutionary consequences of hybridization are nontrivial in plants and animals. For example, the crucial role of ecological selection in the Cuatro Ciénegas Cyprinodon system supports broader arguments by Gross & Rieseberg (2005), who provide compelling evidence that ecological selection was an important factor in homoploid hybrid speciation of three Helianthus taxa; these authors also suggest that ecological selection may have been crucial to the origin of a variety of plant and animal hybrid species, including the fish Gila seminuda (DeMarais et al. 1992). Similarly, Mavárez & Linares (2008) present evidence that in addition to fishes, homoploid hybrid speciation has occurred in several other animal groups, including butterflies, ants, flies and cladocerans, though they argue that because ecological selection does not have a general role in reproductive isolation other behavioural or genetic factors are required to explain establishment of hybrid species. Furthermore, several species associated with adaptive radiation, such as Darwin’s finches, multiple African cichlids and Lake Baikal sculpins (Seehausen 2004 and references therein) display cytonuclear discordance at basal phylogenetic branches, a discrepancy that is thought to have arisen in concert with ancestral introgressive hybridization.

Most studies have lacked clear connection between the circumstances and evolutionary products of hybridization. Meyer et al. (2006), however, approached such a synthesis in a study of the hybrid species Xiphophorus clemenciae, where they linked results from mate choice experiments with laboratory produced hybrids between the putative parental species to observed cytonuclear discordance in the phylogenetic placement of this species. For this, they proffered female preference for sword length as the selective mechanism that led to the formation of the hybrid species X. clemenciae. Although this scenario is plausible, clear demonstration is lacking, as initial conditions are wholly unknown and reasonable alternatives can be invoked, including simple mitochondrial replacement in a parental form. The case of Cuatro Ciénegas Cyprinodon stands in stark contrast to most systems studied to date—particularly in animals—because it represents a single system for which there is a clear connection between the importance of exogenous selection in a contemporary hybrid zone and long-term evolutionary diversification that emerged from ecological selection on introgressed adaptive variants.

Conclusions

The spatio-temporal relationships between environmental and genetic variation in the Rio Churince hybrid zone extend recent evidence that exogenous selection can be a crucial component to the structure of animal hybrid zones. Furthermore, results from this study and those by Tobler & Carson (2010) establish a critical link between contemporary hybridization and evolutionary diversification in species that share a demonstrably long history of genetic interaction. A substantial body of recent studies gives credence to arguments by Arnold (1997, 2006) and others that exogenous selection in animal hybrid zones has been underappreciated. The apparent absence of exogenous selection in many well-studied systems could, in fact, be an artefact of inadequate temporal breadth of prior investigations. To obtain a sufficiently comprehensive understanding of selection in animal hybrid zones, it is crucial that the dynamics of hybridization are not only characterized for disparate systems and interacting taxa but also across spatio-temporal scales most pertinent to the fitness of hybrid individuals. Comparison of such studies will help determine whether hybrid zones represent broadly significant crucibles of animal evolution.

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References


Data accessibility

DNA sequences: GenBank accession numbers EU086365–EU086370.

GPS coordinates for sample locations: Table S4 (Supporting information).

Environmental and genotypic data: DRYAD entry doi: 10.5061/dryad.1h8402s6.

Supporting information

Additional supporting information may be found in the online version of this article.

Table S1 Results of principal component analysis on environmental variables.

Table S2 Counts of parental, backcross, intermediate, and putative F1 genotypes for each population (summed over all collection periods).

Table S3 Levels of admixture in the Churince hybrid zone, by proportion of alleles specific to C. atrorus P(a).

Table S4 GPS coordinates of Sites Chu01-17.

Fig. S1 Variation in mean environmental conditions along axis 1 (circles) and 2 (squares) of 17 populations across seasons and years.

Fig. S2 Seasonal variation in specific conductance (micro-Siemens) and temperature (°C) across the Rio Churince system (sites Chu01-17).

Appendix S1 Characterization of Spatio-temporal environmental variation in the Rio Churince system.

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E.W.C. primarily investigates population genetics, evolutionary ecology, and hybridization of fishes, with a particular emphasis on questions related to spatial and temporal variation. The research of M.T. focuses on relationships between environmental variation and evolutionary ecology of fishes. R.J.A. conducted his senior thesis on hybridization in pupfishes at Arizona State University. T.E.D. uses a variety of molecular markers to investigate the origin and maintenance of organismal diversity. W.L.M. was a uniquely gifted scholar of and relentless advocate for the conservation of fishes, especially desert species; his contributions have been invaluable.

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Senn HV, Barton NH, Goodman SJ, Swanson GM, Abernethy KA, Pemberton JM (2010) Investigating temporal changes in hybridization and introgression in a predominantly bimodal hybridizing population of invasive sika (Cervus nippon) and native red deer (C. elaphus) on the Kintyre Peninsula, Scotland. Molecular Ecology, 19, 910–924.


