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Spatio-temporal variation in parasite communities maintains diversity at the major histocompatibility complex class IIβ in the endangered Rio Grande Silvery Minnow.

Megan J. Osborne[1], Tyler J. Pilger[1], Joel D. Lusk[2] and Thomas F. Turner[1],

[1]Department of Biology and Museum of Southwestern Biology

MSC 03-2020, University of New Mexico,

Albuquerque, New Mexico, 87131, USA

[2]U. S. Fish and Wildlife Service

New Mexico Ecological Services

Albuquerque, New Mexico, 87113.

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Corresponding author: Megan J. Osborne, Department of Biology and Museum of Southwestern Biology, MSC 03-2020, University of New Mexico, Albuquerque, New Mexico, 87131, USA. FAX: 1 5052770304; email: mosborne@unm.edu

Running title: MHCIIβ diversity in Rio Grande silvery minnow

Abstract

Climate change will strongly impact aquatic ecosystems particularly in arid and semi-arid regions. Fish-parasite interactions will also be affected by predicted altered flow and temperature regimes, and other environmental stressors. Hence, identifying environmental and genetic factors associated with maintaining diversity at immune genes is critical for understanding species' adaptive capacity. Here we combine genetic (MHC Class IIβ and microsatellites), parasitological and ecological data to explore the relationship between these factors in the remnant wild Rio Grande silvery minnow (*Hybognathus amarus*) population, an endangered species found in the southwestern United States. Infections with multiple parasites on the gills were observed and there was spatio-temporal variation in parasite communities and patterns of infection among individuals. Despite its highly endangered status and chronically low genetic effective size, Rio Grande silvery minnow had high allelic diversity at MHC Class IIβ with more alleles recognised at the presumptive DAB1 locus compared to the DAB3 locus. We identified significant associations between specific parasites and MHC alleles against a backdrop of generalist parasite prevalence. We also found that individuals with higher individual neutral heterozygosity and higher amino acid divergence between MHC alleles, had lower parasite abundance and diversity. Taken together, these results suggest a role for fluctuating selection imposed by spatio-temporal variation in pathogen communities and divergent allele advantage in maintenance of high MHC polymorphism. Understanding the complex interaction of habitat, pathogens and immunity in protected species will require integrated experimental, genetic and field studies.

Introduction

A vital component of the adaptive immune response in vertebrates are genes of the highly polymorphic major histocompatibility complex (MHCIIβ) (e.g. Klein 1986). The MHCIIβ genes present parasite-derived antigens to T lymphocytes so fluctuating parasite-mediated

selection has been proposed as one mechanism maintaining polymorphism at MHC IIβ (e.g. Meyer & Thompson 2001; Hill 1991; Hedrick *et al*. 1987). Other explanations for high diversity at MHC include balancing selection by heterozygote advantage (e.g., Doherty & Zingernagel 1975) and frequency dependent selection (e.g., Bodmer 1972) imposed by host-parasite co-evolution. Pathogen species and abundance vary in space and time through biotic and abiotic factors and stochastic extinction/colonization events. The fluctuating selection hypothesis proposes that this variation in parasite communities causes differential directional selection intensity, so alternative MHC IIβ alleles will be favored at different points in space and time (Meyer & Thompson 2001; Spurgin & Richardson 2010). Hedrick (2002) demonstrated that MHC diversity could be maintained by fluctuating selection in the absence of heterozygote or rare-allele advantage provided that resistance alleles were dominant. Spurgin and Richardson (2010) summarized two predicted relationships of pathogen and MHC IIβ diversity. Specifically, associations between (i) specific pathogens and MHC IIβ alleles indicates either fluctuating selection or rare-allele advantage and (ii) MHC IIβ heterozygosity and pathogen abundance or richness is suggestive of heterozygote or divergent allele advantage. Hence, infectious disease and parasites can be strong selective forces on their host populations (e.g. Brüniche-Olsen *et al*. 2016; Wegner *et al*. 2003). For example, Eizaguirre *et al*. (2012) showed that varying parasite selection could cause rapid evolutionary change in experimental populations of stickleback.

The immune systems of fishes are highly temperature dependent (Ellis 2001; Dittmar *et al*. 2014), hence fish-parasite interactions may be strongly affected by variable water flow, changing temperature regimes, and by other forms of environmental stress (Lafferty & Holt 2003; MacNab & Barber 2011; Lõhmus & Bjorklund 2015; Brunner & Eizaguirre 2016; Scharsack *et al*. 2016). Recently, Marcogliese *et al*. (2016) demonstrated that changes in

precipitation affected both parasite abundance and species richness on a freshwater fish (spottail shiner *Notropis hudsonius*). Variable environmental conditions that alter disease pressures may thus pose an acute risk to endangered aquatic species which may lack the genetic diversity, particularly at immune genes, necessary to battle infections and to adapt to emergent pathogens (Reed & Frankham 2003; Saccheri *et al*. 1998; Westemeier *et al*. 1998). This is an issue of critical concern to conservation biologists.

Numerous population level studies of MHC have been conducted in salmonids and other fishes (e.g Landry & Bernatchez 2001; Dionne *et al*. 2007; Dionne *et al*. 2009; Tobler *et al*. 2014) but few involving freshwater minnows and carps of the hyper-diverse cyprinid lineage (Family Cyprinidae – over 2400 species [Nelson 2006]). Almost half of North America's cyprinids are considered imperiled (Jelks *et al*. 2008) and diminished MHC variation can place species at increased risk. Here we characterize population level variation at MHCIIβ in the North American cyprinid, Rio Grande silvery minnow. This species is protected under the Endangered Species Act (U.S. Department of Interior 1994), and now occupies < 5% of its historic range in the Rio Grande, New Mexico. The Rio Grande is highly modified and heavily impacted by irrigation and water diversion for human use. Water extraction contributes to seasonal river intermittency, and consequently fish become confined to pools where they are exposed to multiple stressors including degraded water quality (e.g. increased temperatures and decreased dissolved oxygen) and increased competition and predation. High fish densities can increase exposure to pathogens and exacerbate susceptibility to infection through heightened levels of stress (e.g. Dittmer *et al*. 2014). Almost two decades of demographic monitoring has shown that densities of the short-lived Rio Grande silvery minnow can vary by orders of magnitude from year to year (Dudley *et al*. 2014). Since 2002, the Rio Grande silvery minnow population has been supported to varying degrees by

augmentation (Osborne *et al* 2012) to prevent extinction. We have tracked neutral genetic diversity in Rio Grande silvery minnow (Alò & Turner 2005; Turner *et al*. 2006; Osborne *et al*. 2012) for 17 consecutive years; providing crucial information about demography, genetic effective size, and the effects of population augmentation. Data from neutral loci has also shown that over the species current range, there is no population structure due to the drifting nature of Rio Grande silvery minnow eggs (Osborne *et al*. 2012). However, these studies do not allow for specific predictions about whether species harbor genetic diversity needed to respond to immunological stressors.

Previous characterization of MHCIIβ in Rio Grande silvery minnow showed that like other cyprinids, it exhibits a duplicated paralogous MHCIIβ region that is comprised of several distinct allelic groups (putative loci) DAB1/DAB2 and DAB3/DAB4 (e.g. van Erp *et al* 1996; Ono *et al*. 1992; Dixon *et al*. 1996; Osborne *et al*. 2011). We compared levels of MHCIIβ diversity at these allelic groups to information obtained from 'neutral' microsatellite loci to place MHCIIβ results into a demographic and evolutionary context. We also characterized the parasite community in two temporal collections (2006 and 2008) from six localities encompassing the remnant Rio Grande silvery minnow population. Environmental variables, genetic and parasite data were then combined to explore the relationship between these factors. If fluctuating selection is shaping diversity at MHC, then we would expect to see differences in spatial and temporal abundance and species of pathogens, resulting in different alleles being selected for at different points in time/space. Together these data permit an integrated study that assesses the interplay of parasitic infection and the potential for adaptive immune response in a fish that inhabits a variable riverine environment in the semiarid southwestern United States.

Methods

Study Sites

The study area was the Rio Grande in central New Mexico, USA (Figure 1). This stretch of the river is characterized by high inter-annual and seasonal variability in flow and water temperature. Sampling sites were selected in association with the water quality program conducted by the New Mexico Environment Department (NMED) and included the following: Site 1: Bernalillo (upstream of the City of Albuquerque), Site 2: Alameda (below Albuquerque's urban storm water runoff return), Site 3: Los Padillas (downstream of Albuquerque's Southside Water Reclamation Facility discharge), Site 4: near Los Lunas (29 km downstream of Albuquerque), Site 5: La Joya (ca. 10 km upstream of San Acacia Diversion Dam) and, Site 6: ca. 6 km downstream of San Antonio. These sites spanned 171 river km encompassing almost the entire current distribution of Rio Grande silvery minnow. Water quality parameters were measured at the location of every seine haul conducted at each site and sampling event using handheld multi-probe meters (YSI Environmental, Model 556, Yellow Springs, Ohio) to record water quality parameters including water temperature, dissolved oxygen (milligrams per liter [mg/L]), percent oxygen saturation and specific conductivity (microSiemens per centimeter [µS/cm]). For subsequent analyses, water temperature, dissolved oxygen (milligrams per liter [mg/L]), percent oxygen saturation and specific conductivity were averaged for each site and year. Discharge (m^3 per sec^{-1}) was obtained using a single cross-sectional channel transect at each site and sampling event.

Fish sampling and examination

This species is subject to population supplementation with captive reared and/or bred fish (Osborne et al. 2012). All supplemented individuals are marked with a visible implant elastomer (VIE) tag prior to release. Only untagged i.e. 'wild' fish were sampled for this

study. Fish were collected by seining (3 m wide x 2 m deep, 3.2 mm mesh) over a one-week period in July (summer) 2006 and 2008. Following collection, fish were transported live to the laboratory for immediate processing. Plastic transport bags were filled with 3 L of river water; the remaining volume of the bag was inflated with pure oxygen. No additional salts or additives were used. To investigate diversity and abundance of parasites among sampling sites, up to 30 individuals per site were examined. Fish were euthanized with an overdose of MS-222 (tricaine methanesulphonate) prior to parasite examination. Examination for external parasites was conducted using wet mounts from gill clippings from each fish. Gill clips were collected with necropsy scissors and placed on microscope slides with a drop of distilled water and covered with cover glasses. Wet mounts were examined at 40x, 100x, 200x, and 400x magnifications using a compound light microscope. Parasites were identified to the species level when possible and enumerated for each sample. Following examination, gill tissue was collected and preserved in RNA*later*® (Ambion) from the first 20 individuals examined for parasitic infections from each collection locality in 2006 and from 30 individuals per locality in 2008 and stored at -80°C. If parasites could not be identified to species level, the taxonomic family, class and/or genus was recorded. Unavoidably, different personnel conducted parasite examinations in 2006 and 2008. Due to high prevalence and abundance of parasites (particularly *Ichthyobodo*) during 2006, and time constraints for examining fish and for preserving RNA in gill tissue, ~25 % of total parasite counts on individuals were estimated by collectors as being greater than a value that they counted up to. For example, an individual fish with greater than 100 parasites, was indicated as 100+. Hence, for the 2006 data we used the last number counted (e.g. for a count of n = 50+ we used n = 50). In 2008, all parasites were counted to a maximum of 100. Two individuals had more than 100 parasites in 2008 (n = 100+). As for 2006, 2008 counts were truncated to the last value counted. Despite differences in counting procedures between years, we do not

expect this to influence further analyses because overall parasite abundances were much higher in 2006 compared to 2008. The significance of results of downstream analyses (that used abundance data) are conservative because of the manner in which we truncated counts.

Molecular Methods

Microsatellites

A small piece of caudal fin was removed from each fish following parasitological analysis. Fin clips were preserved in 95% ethanol for subsequent genomic DNA isolation. Whole fish sampled for parasites were frozen and deposited at the Museum of Southwestern Biology Division of Fishes (ACC2006-VII:31). Total nucleic acids were extracted from fin clips from 30 individuals per locality using proteinase-K digestion and organic extraction methods (Hillis *et al.* 1996). Individuals were genotyped at nine polymorphic microsatellite loci: *Lco1*, *Lco3*, *Lco6*, *Lco7*, *Lco8* (Turner *et al*. 2004); *Ca6* and *Ca8* (Dimsoski *et al*. 2000); and *Ppro118* and *Ppro126* (Bessert & Orti 2003) as described in Osborne *et al*. (2012). Genotype data was obtained using GeneMapper Version 4.0 (Applied Biosystems).

MHC Class IIβ Expression

RNA isolations from gill tissue and cDNA synthesis were conducted as described in Osborne & Turner (2011). cDNA was used rather than genomic DNA as we were interested in the number of expressed MHC alleles. Individual cDNA samples were screened for the presence of each MHCIIβ allelic group using group specific primers to maximize characterization of allelic variation (Osborne & Turner 2011). Two sets of primers and PCR conditions were used to amplify DAB1/DAB2 as this locus is comprised of two distinct groups of distinct alleles (Osborne & Turner 2011). It is not clear which alleles correspond to DAB1 and DAB2 or which alleles belong to DAB3 and DAB4 so we refer to them collectively as DAB1 and

DAB3, respectively. DAB1 alleles are labeled as Hyam-DAB1 or Hyam-DAB1a based on which primer set was used to amplify them. Hyam-DAB1 [*MHC Ex2F* and *Lc1R*], DAB1a [*MHC Ex2F* and *Lc2R*], Hyam-DAB3 [*MHC Lc4F* and *Lc4R*] alleles were amplified (1X Genescript Taq polymerase buffer, 3mM $MgCl_2$, 125 μM dNTPs, 0.35 μM each primer, 0.375 units Taq polymerase) under the following PCR cycling parameters: one denaturation cycle of 92°C for 2 mins followed by 30 cycles of 90 °C for 20s, 53°C (DAB1) or 56°C (DAB1a or 58°C (DAB3) for 20 s, 72°C for 20s and a final extension step of 72°C for 10 minutes. Individuals that failed to amplify using the primer combination given above were amplified with *MHC Ex2F* or *MHC Lc4F* in conjunction with *MHC-Ex3R* with the above conditions. Use of four sets of PCR primers maximized the characterization of MHC Class II variation. We do acknowledge that it is possible that not all MHC alleles present were amplified, either because they were not expressed or PCR primers were not optimal for additional variants.

Single Stranded Conformational Polymorphism Analysis

Samples that were positive for each of the allelic groups were amplified in 15μL volumes (using the above conditions). Five microliters were used to check for successful PCR amplification and 10μL of sequencing stop solution (95% formamide, 20 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol FF) was added to the remaining 10μL of PCR product. Samples were denatured at 93°C for 5 minutes. Fifteen microliters of each sample was loaded on a non-denaturing polyacrylamide minigel (5% acrylamide for allelic groups Hyam-DAB1a and Hyam-DAB3 and 6% for Hyam-DAB1) (with 50% glycerol). Samples with known genotypes were included to assist in genotype designation for unknown samples. Electrophoresis was carried out on a BioRad minigel system (Protean® II xi Cell) in 1X TBE at approximately 4°C for 4 hours for Hyam-DAB1a and 4.5 hours for groups Hyam-DAB1

and Hyam-DAB3. Polyacrylamide gels were stained in ethidium bromide and visualized on a UV transilluminator.

Gel slices from each variant were removed from the gel and resuspended in 30μL of deionized water and 30 μL of TE and incubated at 37°C, shaking for 2-3 hours. Samples were centrifuged for 20 minutes at 30,000 RPM and the supernatant removed and placed in a new 1.7 ml centrifuge tube. After standard ethanol precipitation samples were resuspended in 50 μL of water. One microliter was used as a template in PCR reactions for sequencing using conditions listed above. PCR products were purified using Marligen PCR cleanup kit and sequenced using ABI Big Dye Terminator kit V.1.1. Sequencing was conducted on an ABI Prism 3130 capillary sequencer (Applied Biosystems). For every individual, each sequence variant was sequenced at least twice. Sequences were visualized and checked using Sequencher Version 4.6 (Gene Codes Corp) and aligned manually using Se-Al Version 2.0a11 (http://evolve.zoo.ox.ac.uk/software.html?id=seal).

To visualize the relationship among MHCIIβ alleles Bayesian phylogenetic analysis was conducted using MrBayes (Ronquist & Huelsenbeck 2003) as implemented on the CIPRES portal with the GTR + G + I model of nucleotide substitution (selected as the most appropriated model of sequence evolution by jModeltest [Posada 2009]). We ran the analysis for 10 x 10^6 generations, sampling the Markov chain every 1000 generations. This produced 10,000 trees of which the first 1,000 were discarded as burn-in. Three replicates were conducted to ensure stability across runs and a 50% majority rule tree was produced. Node support was determined by posterior probabilities obtained for the majority-rule consensus tree. We included previously cloned and sequenced Rio Grande silvery minnow alleles (Osborne & Turner 2011), and as well as other cyprinid taxa to show the conservation of the

DAB1 and DAB3 loci across the evolutionary history of the family Cyprinidae. Outgroup taxa include longnose dace (*Rhinichthys cataracterae*, EU848571.1-EU848574.1), zebrafish (*Danio rerio*, BC124461.1), Lake Tana barbels (*Barbus intermedius*, AJ507002.1, AJ507001.1), common carp (*Cyprinus carpio*, X95434.1, X95431.1, Z49065.1, EU203666.1), carp bream (AJ811683.1) and European chub (*Squalius cephalus*, HQ595097.1, HQ595133.1) available on Genbank. Accession numbers for Rio Grande silvery minnow sequences are provided in Table S1.

Statistical Analysis

Parasite Communities and Environmental Variables

Ecological analysis of parasite data was performed in R (ver. 3.2.4, R Core Team 2016) using package vegan (Oksanen *et al*. 2015). Parasite communities were analyzed at two levels (i) infracommunity (parasite communities on individual fish) and (ii) the component communities (combined infracommunities among a sample of fish) as defined by Bush *et al*. (1997). First, infracommunity differences across sites and years were examined using non-metric multidimensional scaling (NMDS) on square root transformed parasite abundances for each individual infected by one or more parasite species. To avoid outlier bias, only parasites with > 5% of total abundance were included in analyses. The number of NMDS dimensions were selected by performing separate analyses that included successively larger dimensions until the change in stress values became negligible. We compared dissimilarity of infracommunity composition (Bray-Curtis distance) across individuals within and among sites and years using permutational multivariate analysis of covariance (PERMANCOVA). Standard lengths were measured for each fish (as a measure of body size) and included as a covariate to control for the influence of body size on parasite abundance. Standard lengths were transformed as natural logarithm to better approach normality. Separate

PERMANCOVAs were also performed for each year to investigate if infracommunities varied similarly across years.

In addition to compositional resemblance, we investigated variation of infracommunity diversity and evenness across sites and years. We calculated an index of parasitization (I_{PI}) following Kalbe *et al*. (2002), as well as Shannon diversity (H) and evenness (J) for each individual. Note that H and J could only be calculated for individuals infected by at least one and two parasite taxa, respectively. Index of parasitization standardizes individual parasite loads across parasite taxa that occur at different ranges of intensity and combines measures of richness and abundances on an individual fish. Variation in diversity metrics were analyzed using separate ANCOVAs with site and year as factors and natural log of standard length as covariate.

Parasite infracommunities are not independent with respect to environmental variables collected at a given site and year, thus we evaluated the relationship of environmental factors and parasites at the site level, i.e., component community. We first assessed correlations among environmental variables and proportion of infected individuals at a site using the Pearson correlation coefficient. Only habitat variables without missing data points were used and included discharge, temperature, pH, and specific conductivity. We considered correlation coefficients of > 0.7 or < 0.7 as strongly correlated. Of the habitat variables, only discharge required transformation with natural logarithm to approach normality. Next, we used an ordination approach to evaluate changes in component community, i.e, combined abundances of each parasite taxon obtained from all fish at a sample location. Correspondence between parasite component community composition and site habitat variables was analyzed using principal coordinates analysis (PCoA). Component community

dissimilarity was quantified using Bray-Curtis distance on square-root transformed abundances. Habitat variables and data transformations used in the PCoA were the same as those used for Pearson correlations.

Neutral (microsatellite) and MHC Molecular Diversity and Divergence

Microsatellite data were analyzed as outlined in Osborne *et al*. (2012). For each microsatellite locus and population, allelic richness (A_R), mean number of alleles (MNA) and inbreeding coefficients (F_{IS}) were obtained using FSTAT version 2.9.3.1 (Goudet 1995). Allelic richness for microsatellites and MHC (N_{ac}) was calculated using the methods described in Petit *et al.* (1998) and implemented in the program Contrib.

We compared patterns of neutral diversity (microsatellites) with MHCIIβ diversity across individuals to test if variation at MHCIIβ was consistent with neutral expectations. We used the program GENHET (Coulon 2010) to calculate proportion of heterozygous loci for each individual (PHt) to represent neutral genetic variation. Individual MHC diversity was represented as the number of alleles expressed per individual and the maximum amino acid divergence (amino acid p-distance calculated in MEGA Vers. 5.1 [Tamura *et al*. 2011]) among alleles. Individual heterozygosity (PHt) and MHCIIβ allelic diversity values were z-transformed in Sigmaplot (Vers. 11) to standardize diversity scores across sites. Spearman rank correlations were used to evaluate the relationship between microsatellites and MHCIIβ diversity at the individual level.

MHC-Selection

The program PAML Vers 4.9a (Yang 2007) was used to identify codons under positive or purifying selection. A phylogenetic tree of MHCIIβ alleles was obtained using MrBayes assuming the GTR + G + I model. The algorithm was implemented on the CIPRES portal (Miller *et al*. 2010) as described above (but without the inclusion of outgroup species). The resulting 50% majority-rule consensus tree was used as a prior for CODEML. We compared the M1a (nearly neutral evolution) with the M2a (positive selection) model and the M7 (β) with the M8 model (β and ω) using a likelihood ratio test (LRT). The LRT statistics were compared to a chi-square distribution with two degrees of freedom. Posterior Bayesian probabilities were calculated using the Bayes Empirical Bayes (BEB) method (Yang 2005) implemented in PAML. This analysis was conducted for all alleles across year. We also analyzed Hyam-DAB1 and Hyam-DAB3 allelic lineages separately (years combined) to determine if different codons were identified as subject to selection among lineages.

Associations between expression of MHCIIβ alleles and infection of each parasite taxa were tested in order to assess whether there was evidence of fluctuating selection. Over dispersion and an excess of zeros, characteristic of parasite count data, can be modeled using zero adjusted models (Chipeta *et al*. 2014). Thus, we opted to use zero-inflated negative binomial (ZINB) models to assess associations between specific alleles or groups of alleles with abundance of each parasite taxa. Each ZINB model consisted of a count model testing for association between presence/absence of MHCIIβ alleles (present in > 5% of individuals) and untransformed parasite counts per individual, and a logistic model where we used site, year, their interaction, and natural log of body size to model excess zeros. Over dispersion of count data was tested by comparing ZINB models with models assuming a Poisson distribution (rather than negative binomial) using likelihood ratio tests (Zuur *et al*. 2009). For each

parasite taxon, we compared nested models, dropping variables from the logistic component one at a time, using likelihood ratio tests to find the best model to account for excess zeros. ZINB analyses were performed using the pscl package (Jackman 2015) in R.

Association between MHC Class IIβ and parasite diversity

We evaluated the relationship between diversity at MHCIIβ and degree of parasitization at an individual and site level. At the individual level we used general estimating equations (GEE) that are commonly used to analyze longitudinal or clustered data by specifying a covariance matrix that accounts for correlation within sample localities (Hanley *et al*. 2003). Separate GEE models were used with response variables including parasite richness, natural log of parasite abundance, and I_{PI} assuming a normal distribution with identity link functions and data clustered within each year by site combination. Predictor variables for each model included number of MHCIIβ alleles per individual, genetic distance (amino acid p-distance calculated in MEGA Vers. 5.1 [Tamura *et al*. 2010]) between most divergent alleles within an individual, and individual microsatellite heterozygosity (PHt). To account for differences in exposure intensity that could result from individuals with different body sizes, we included natural log transformed standard length as an offset. We used an exchangeable correlation structure with a robust parameter estimator implemented in the geepack package (Højsgaard *et al*. 2006) in R. Finally, at the site level, we regressed mean richness, abundance, and I_{PI}, against mean PHt, number of MHCIIβ alleles, and amino acid distance.

Results

Body size of fish collected for this study in 2006 ranged from 30 mm to 85 mm (mean= 55.98 mm, SE=0.66 mm) and in 2008 ranged from 21 mm to 73 mm (mean 38.87, SE= 1.08). In 2008, body size was bi-modally distributed indicating the presence of two age classes (age 0

and age 1) in the collections. Body size differed significantly across years (Kruskal-Wallis chi-square = 129.8, $p < 0.001$) and was included as a covariate in subsequent across-year statistical analyses. Densities of Rio Grande silvery minnow differed by an order of magnitude between 2006 and 2008 with mean CPUE across all population monitoring sites (n=20) of 0.037 fish per m^2 (SEM=0.011) in 2006 and 0.350 fish per m^2 (SEM=0.098) in 2008 (American Southwest Ichthyological Researchers, R. Dudley pers. comm.*)*.

Spatial and temporal variation in parasite communities

The gills of 326 Rio Grande silvery minnow were examined. Nine parasite taxa were identified on 228 infected individuals. The proportion of infected individuals was highly variable across sites and years ranging from 65% to 95% in 2006 and 10% to 100% in 2008 (Table 1). The four most abundant taxa were ectoparasitic trichodinids and *Ichthyobodo*, and the ectocommensals *Apiosoma* and *Cryptobia*. We only included these four taxa in analyses as they accounted for 98% of total parasite abundance. *Epistylus* was occasionally observed in both years and *Ambiphyra, Tremoda*, *Trichodinella* and *Ichthyophthirius* were recorded 2008 at multiple sites but on very few individuals.

Parasite infracommunities primarily segregated by year with 2006 characterized by heavy *Ichthyobodo* infection among individuals whereas 2008 infracommunites included greater number of taxa (Figure 2). However, overall infection by parasites was lower in 2008 (mean proportion of infected individuals = 0.66, mean $I_{pI} = 1.04$) compared to 2006 (mean proportion of infected individuals = 0.79, mean $I_{pI} = 1.95$) (Table 1). PERMANCOVA was used to evaluate factors explaining variation among infracommunities. Body size accounted for 12% of community dissimilarity (Table 2). The largest component of variation was residual (64%), suggesting that infracommunities were highly variable among individuals.

Site, year, and their interaction accounted for 23% of infracommunity dissimilarity. The interaction between site and year was further investigated by performing PERMANCOVAs separately for each year (Table 2). Whereas site accounted for a significant 26% community dissimilarity in 2006, site only accounted for marginally significant 8% in 2008.

Mean I_{PI}, H and J were similar across years ($I_{PI\ 2006}$ = 1.81, $I_{PI\ 2008}$ = 1.22; H_{2006} = 0.25, H_{2008} = 0.32; J_{2006} = 0.73, J_{2008} = 0.71; Figure 3). Univariate ANCOVA models identified a significant effect of site (P = 0.003) and year (P = 0.002) with a marginally significant effect of body size (P = 0.058) on I_{PI} ($F_{12,210}$ = 3.003, P < 0.001). Whereas both Shannon diversity and evenness exhibited significant site by year interactions (P < 0.001 and P = 0.017, respectively) only the ANCOVA comparing H was a significant model ($F_{12,210}$ = 3.526, P < 0.001).

Associations among environment and parasite communities

Mean water temperature at sampling localities was higher in 2006 than in 2008 (Table 1). In 2006, discharge values varied across sites with a maximum of 77 m^3 per sec^{-1} at the most upstream site to less than 1.46 m^3 per sec^{-1} at the three downstream sites (site 4-6). In 2008, discharge was relatively high across all sites ranging from 12.2 to 72 m^3 per sec^{-1}. Generally, environmental variables were not intercorrelated except for temperature with discharge (Pearson's r = -0.79) and specific conductivity (r = 0.72). Proportion of infected individuals at a site was not strongly correlated with any environmental variables. Environmental drivers of parasite component community composition were assessed using PCoA. Although environmental measures varied substantially across sites and years, neither discharge, temperature, pH nor specific conductivity significantly explained variation in Bray-Curtis

distances among component communities. Thus, we did not present a visualization of this ordination.

Neutral (microsatellite) and MHC Molecular Diversity and Divergence

There were 22 departures from Hardy-Weinberg equilibrium from 108 comparisons after sequential Bonferroni correction (Rice 1989). Eleven total deviations occurred at *Ppro118* and *Lco8*, two deviations were observed at *Ca6* and *Lco7*, three at both *Lco3* and *Ca8* and there was one deviation at *Lco1*. Two loci (*Ppro126* and *Lco3*) conformed to HWE in all comparisons. Departures were explained by a deficiency of heterozygotes. There was no evidence of linkage disequilibrium between loci after Bonferroni correction (Rice 1989). Microsatellite gene diversity, observed heterozygosity and allelic richness was similar across sites and years (range $H_e = 0.82 – 0.87$; $H_O = 0.62 – 0.74$; $A_R = 9.2 – 10.3$) (Table 3).

Sequence data (274 bp) encompassing 91 codons of the exon two region of MHCIIβ region was obtained from 252 individuals (Table 3). MHCIIβ alleles could not be amplified from 4 and 23 individuals in 2006 and 2008, respectively. Failure to amplify MHC from these individuals was not likely a result of the PCR primers because successful amplification of MHC occurred using genomic DNA as a template, suggesting either degradation of RNA in these samples or absence of expression of these alleles. Across sampling events, total number of observed MHCIIβ alleles per event ranged from 12 (site 5, 2006) to 25 (site 4, 2008) and 6.17 (site 5, 2006) to 9.65 (site 3, 2008) once we accounted for differences in sample size (Table 3). Mean number of alleles expressed per individual also varied across sampling events (range 1.17 – 2.15).

Sixty-eight unique alleles were identified that could be assigned to two highly divergent allelic groups identified previously (Hyam-DAB1 and Hyam-DAB3) (Osborne & Turner

2011). Eleven newly identified alleles belonged to a well-supported monophyletic clade corresponding to Hyam-DAB3 which was further split into two groups (likely DAB3 and DAB4 but it is not clear which alleles correspond to which locus). The first contained previously described Hyam-DAB3 alleles (D, S, X, T) and Hyam-DAB3*13 (identified here) which was sister to a clade containing longnose dace (MHCIIB*I-IV), zebrafish and common carp DAB3 and DAB4 alleles (Figure S1). The second well-supported DAB3 group comprised exclusively Rio Grande silvery minnow sequences. The DAB1 alleles formed two well supported clusters of more than 20 alleles each (posterior probability = 0.83, 0.84) (likely corresponding to DAB1 and DAB2) and nine alleles could not be assigned to one or the other. Conserved features of MHCIIβ were identified in all allelic lineages including cysteine residues and the putative N-linked glycosolation site. Eighteen DAB1 alleles had a single codon deletion at position 78 (numbering based on Osborne et al. 2011). Only 11 alleles were present in more than 5% of individuals (Table S1). Based on comparisons to previously published MHCIIβ data (Osborne & Turner 2011), individuals could be placed into the following groups 1) individuals that expressed one or multiple alleles from a single allelic group [DAB1, or DAB3]; 2) individuals that expressed one or multiple alleles from multiple allelic groups [DAB1 & DAB3] and 3) individuals that did not express alleles (or expression was not detected) in any group. The maximum number of alleles detected per individual was four. Individual expression of DAB3 alleles roughly doubled from 2006 (10.5%) to 2008 (23%). Within this allelic lineage, Hyam-DAB3*06 was the most frequently encountered allele in each year. No individuals were observed that had more than one DAB3 allele in any year.

Evidence of Selection

The null models (M1a and M7) were rejected in favor of those accounting for positive selection (M2a and M8) at the codon level. Across allelic lineages, sixteen positively selected codons were identified (Table 4). Separate analysis of Hyam-DAB1 and Hyam-DAB3 alleles identified 17 and 18 codons respectively under positive selection; seven of these codons were shared between DAB1 and DAB3.

Association between MHC Class IIβ *alleles and individual parasite taxa*

We used ZINB models to identify associations between MHCIIβ alleles or groups of alleles and individual parasite taxa (count model) while accounting for excess zeroes which may be due to differences in component communities among locations/years or detection bias due to fish body size (logistic model). Of the 68 identified MHCIIβ alleles, 11 occurred at high enough frequencies and enough individuals were infected by *Ichthyobodo* and *Trichodina* to be include ZINB models for these parasites, whereas only 7 and 5 alleles were present in enough individuals infected by *Cryptobia* and *Apiosoma*, respectively to include in ZINB models. Likelihood ratio tests comparing ZINB models with their Poisson counterparts were significant across the four parasite taxa (all $X^2 > 540$, df = 1, all $P < 0.001$) indicating over-dispersion in the non-zero count data. Seven unique alleles representing MHCIIβ groups DAB1, DAB1a, and DAB3 were significant ($P < 0.05$) or marginally significant ($P < 0.1$) predictors for the presence of parasites (Table 5). Both alleles Hyam-DAB1*12 and Hyam-DAB1*13 were associated with two of four parasite taxa (*Cryptobia* and *Apiosoma*), whereas the other five alleles were each associated with a single species. *Ichthyobodo* had significant associations with the greatest number of alleles (n = 3), whereas *Trichodina* was associated with only one allele. Among parasite taxa, logistic models of excess zeros were best explained by natural log of body size for *Ichthyobodo* ($X^2 = 59.2$, df = 10, $P < 0.001$) and

Trichodina (X^2 = 81.6, df = 10, P < 0.001). Although site and year were not significant in logistic models for *Cryptobia* and *Apiosoma*, we were not able to test the effects of body size due to uneven infection of these two parasite taxa across sites and years (e.g., no individuals were infected with *Apiosoma* at sites 1 – 3 in 2006 and only one individual was infected at site 6).

*Association between genetic (neutral and MHC*IIβ*) and parasite diversity*

To ascertain if diversity at MHCIIβ conformed to neutral expectations we evaluated the correlation between individual diversity at MHCIIβ with individual microsatellite diversity (PHt). Neither the standardized number of MHCIIβ alleles nor the standardized maximum amino acid divergence between them were correlated with standardized PHt (number of MHCIIβ alleles P = 0.846; amino acid divergence P = 0.937).

We used GEEs to evaluate the relationship between individual levels of infection (measured as richness, natural log of total abundance, and individual parasitization index [I_{PI}]) and neutral diversity (PHt), number of MHC alleles (N_{ac}) and individual maximum amino acid distance. Both I_{PI} and abundance were negatively associated with PHt and amino acid distance (P values ranged from 0.028 to 0.065; Table 6). However, no effect of number of alleles was observed nor was parasite species richness influenced by genetic diversity (MHCIIβ or neutral). At the site level, we found no relationship between parasitism and genetic diversity.

Discussion

We present a comprehensive analysis of genetic diversity of functional MHCIIβ genes (DAB1 and DAB3 alleles) as well as neutral microsatellite loci in the wild population of Rio Grande silvery minnow, alongside characterization of its parasite community and key environmental parameters. We found that Rio Grande silvery minnow were infected with multiple pathogens including ectocommensals and ectoparasites but patterns of infection varied by site and year. Analysis revealed high levels of allelic diversity at MHCIIβ in Rio Grande silvery minnow with more recognized as DAB1 alleles compared to DAB3 alleles. There was also high divergence among allelic lineages. Evolutionary patterns differed between DAB1 and DAB3 allelic lineages with only seven shared positively selected codons between them, suggesting that these are distinct loci with diversified function; consistent with findings in other cyprinids. We identified associations between specific parasites and MHCIIβ alleles consistent with fluctuating selection imposed by spatio-temporal variation in the pathogen community (e.g. Oliver *et al*. 2009) in the Rio Grande. Also, GEE identified a negative association between diversity (PHt and MHCIIβ amino acid distance) and parasite load (abundance and I_{PI}), suggesting that individuals with higher MHC divergence and neutral heterozygosity had lower parasite loads; compatible with heterozygote or divergent allele advantage (Spurgin & Richardson 2010). The observation that individual heterozygosity at microsatellites is associated with lower parasitism is in accord with the idea that more outbred individuals are fitter than more homozygous (or inbred) individuals (e.g. Brown 1997; MacDougall-Shackleton 2005).

Environmental Variables and Parasite Communities

Our results highlight the spatial and temporal variability of abiotic components of riverine ecosystems like the Rio Grande, and the impacts that this has on habitat parameters, fish densities and parasite communities. Variation in natural precipitation patterns, coupled with human alterations to the system, like flow regulation and water extraction result in substantial differences in flow and temperature for both site-within-year and among-year comparisons. For example, in 2006, flows were lower while temperatures were higher across almost all sites and this difference was most noticeable at three downstream sites (Los Lunas, La Joya, San Antonio).

Local parasite community and an individual's infracommunity can be influenced by environmental conditions (e.g. temperature, discharge, pollutants), host parameters including life-history (e.g. body size, longevity, immunity, population size) and parasite features/lifecycle (e.g. Sasal *et al*. 1999). Along with high variability of infracommunities among individuals, we found that the number of infected fish varied enormously among sites and years, which is consistent with the highly stochastic nature of the Rio Grande. In some instances, parasite component communities could be very different across geographically adjacent sites. For example, fish from site 5 (La Joya) had high species diversity and abundance of multiple parasites while the majority of fish from site 6 (San Antonio) were heavily infected *Ichthyobodo* and hence species diversity was low. Also, at the same site the number of infected individuals could differ dramatically between years. However, differences in water temperature and discharge could not account for variation seen in the parasite component communities. Because of the highly variable nature of the system, two years is likely not enough time to evaluate associations between environmental factors and parasite

communities. Additionally, lack of an association may be because changes in water temperature do not affect all parasites similarly. For example, Karvonen *et al*. (2010) showed that increasing water temperature increased prevalence of some parasite species but not others. Furthermore, they found that there was not a uniform effect of temperature across fish age classes (Karvonen *et al*. 2010). Interestingly in 2008, young-of-year were present in collections analyzed here, along with age-1 fish. Spawning of semi-buoyant, non-adhesive eggs in Rio Grande silvery minnow is stimulated by increases in water temperature and stream discharge associated with spring melting of high mountain snowpack (Platania & Altenbach 1998). Hence, the presence of young-of-year in the 2008 sample is explained high spring discharge and associated higher densities of Rio Grande silvery minnow. In contrast, in 2006 young-of-year were virtually absent in our collections due to the lack of spring snowmelt runoff and much lower densities of Rio Grande silvery minnow; likely through a combination of reduced spawning and poor initial survival. Sites where young-of-year fish predominated had high levels of infection (~78-100% infected). Activation of the adaptive immune system is slow (i.e. several weeks in fish and is temperature dependent; e.g. Whyte *et al*. 1987) so presumably young-of-year fish have had less time to mount an adaptive immune response to clear the parasites, than their larger (older) counterparts. The adaptive immune response is energetically costly. For example, Kurtz *et al*. (2006) showed that fish with high levels of MHC expression were in poor condition and had elevated levels of oxidative stress. Also, Wegner *et al*. (2006) found heightened MHC expression in stickleback selected for higher parasite load (i.e. lower innate response). They suggested that an efficient innate immune response may depress overall activation of the immune system; reducing negative impacts (e.g. oxidative stress) of mounting a specific immune response (Kurtz *et al*. 2006). Nonetheless, young-of-year Rio Grande silvery minnow (<20-30 mm) did express MHCIIβ alleles, suggesting that upregulation of adaptive immune genes, although

energetically costly, may provide some benefit. Together these results indicate that the component parasite community and an individual's infracommunity is a product of the complex interaction of factors and identifying a single determinant in a dynamic system such as the Rio Grande is likely unrealistic.

Association between genetic and parasite diversity

Klein & O'Huigin (1994) suggested that the immune system of hosts would be more likely to evolve in response to specific parasites than to generalists. Hence, low host specificity should erode co-evolutionary dynamics and favor a broader spectrum of individual MHC diversity including maximal or optimal diversity and sequence divergence (reviewed in Eizaguirre & Lentz 2010). Also, pathogens must cause significant pre-reproduction mortality to be a strong selective force on MHCIIβ (e.g. Savage & Zamudio 2016). The parasites infecting Rio Grande silvery minnow are considered generalists, so we did not anticipate an association between specific alleles and parasites. However, we found several significant associations between the most common alleles and parasites; consistent with the fluctuating selection hypothesis. Fluctuating selection can maintain diversity at MHCIIβ (Hill 1991) because variation across space and time in the pathogen community will cause fluctuating intensity of directional selection (Spurgin & Richardson 2010). Expression of alleles Hyam-DAB1*04, DAB1*12 and DAB1a*04 were associated with infection by the protozoan *Icthyobodo necator* which can damage gills and skin, reduce respiratory efficiency, decrease condition and cause mortality; with poor environmental and crowded conditions increasing these effects (Noga 2000). There was also a significant association between Hyam-DAB1*13 and Hyam-DAB1*07 and infection with *Cryptobia.* Jones (2001) suggested that some *Cryptobia* spp. have well defined host ranges and that both innate and adaptive immune responses (Jones & Woo 1987; Jones *et al*. 1993) were involved in an individual's response to, and

recovery from, infection. Likewise, infection with *Apiosoma* was related to expression of DAB1*12 and DAB1*13. Both *Epistylus* and *Apiosoma* are ciliated ectocommensals and can become problematic when there are high loads which physically impair respiration. They also make individuals prone to secondary bacterial infections. Hence, the association between DAB1*12 and DAB1*13 and *Apiosoma* could also be a response to bacterial infection. It is not clear however, whether presence of these alleles ultimately enhance or decrease survivorship from infection. For example, Zhang *et al*. (2006) found that particular MHC alleles occurred at higher frequencies in flounder stocks resistant to *Vibrio anguillarum* infection, whereas alternative alleles were found at higher frequencies in flounder stocks susceptible to infection. Interestingly, only a minority (~10% [2006]-23% [2008]) of Rio Grande silvery minnow individuals expressed genes (from gill tissue) belonging to the DAB3 allelic group and the one allele that was sufficiently frequent to test for an association with parasite presence, was not significantly associated with any gill parasite. MHC expression in other tissues associated with the immune response (e.g. gut and thymus; Buonocore *et al*. 2007) may reveal greater expression of DAB3 alleles in response to infection with endoparasites. For example, Seifertová *et al*. (2016) found a positive association between DAB3 alleles and endoparasite abundance. Large numbers of alleles present at low frequencies within Rio Grande silvery minnow, reduces power to test for correlations between parasites and MHCIIβ diversity. For this reason, it is surprising that we see evidence of a relationship between the most common alleles and parasites in Rio Grande silvery minnow. In contrast, carp bream and three-spined stickleback (which also have high numbers of MHC alleles, most of which occur at low frequencies) showed no association between expression of certain MHCIIβ alleles and resistance to specific parasites (Ottova *et al.* 2007; Wegner *et al*. 2003). The associations between MHC alleles and specific parasites identified

here should be examined more closely in an experimental setting where confounding factors associated with field studies can be minimized.

In addition to observing relationships between particular pathogens and alleles, we identified a negative association between diversity (PHt and amino acid distance and parasite load (abundance and I_{PI}) whereby individuals with higher individual heterozygosity (at neutral loci) and higher amino acid divergence between MHCIIβ alleles (highest divergences were between DAB1 and DAB3 alleles) had lower parasite abundance and index of parasitization. Similarly, Rakus *et al*. (2009) found that in common carp, F1 individuals with DAB1-like and DAB3-like genes had higher resistance to *Trypanoplasma borreli* and their data also suggested that heterozygosity at genes other than DAB could affect resistance. Consuegra & de Leaniz (2008) found that progeny of wild Atlantic salmon individuals (freely mating) had divergent MHCIIβ genotypes, and non-infected individuals also had more dissimilar MHC when compared to the progeny of salmon artificially crossed in the hatchery (no mate choice). This suggests a role for disassortative mating (i.e., individuals with dissimilar genotypes mate more frequently than expected under random mating) to maximize MHC divergence among alleles; which makes offspring more resistant to parasitic infections. There may also be functional redundancy of distinct alleles if they are not sufficiently divergent. The association between heterozygosity and lower parasite load and I_{PI} is consistent with a general effect such that neutral heterozygosity is related to an individual's inbreeding coefficient and hence heterozygosity across the genome; including disease resistance loci (MacDougall-Shackleton 2005). Alternatively, there may be a local effect in which the microsatellites used are in linkage disequilibrium with other disease resistance loci (not necessarily MHC) (Hansson & Westerberg 2002; MacDougall-Shackleton 2005).

Differential Functions of DAB1 and DAB3

Like other cyprinids, Rio Grande silvery minnow exhibit a duplicated paralogous MHCIIβ region that is comprised of several distinct allelic groups (loci) DAB1 and DAB3 (e.g. van Erp *et al* 1996; Ono *et al*. 1992; Dixon *et al*. 1996; Osborne & Turner 2011). There are different patterns of evolution between these putative loci that also vary across species. In Rio Grande silvery minnow, we found that <25% of individuals expressed alleles belonging to the DAB3 group with no individual examined here expressing more than one DAB3 allele. Allelic diversity was also lower; with only 13 DAB3 alleles detected compared to >50 DAB1 alleles. The limited allelic variation and increased homozygosity at DAB3 observed in longnose dace (Girard & Angers 2011) are consistent with this results. In common carp, a comparable pattern is observed for *Cyca*-DAB2 in which individuals are often homozygous at that locus or it is entirely absent from genomic DNA (Rakus *et al*. 2009). Likewise, in European chub, the majority of fish only express one copy of the DAB1 allelic group and higher diversity was found at DAB3 when compared to DAB1 (Seifertová & Simková 2011). Despite differences in diversity patterns among species and among these loci/allelic lineages in cyprinids, these ancient lineages have been maintained as functional loci. Wakeland *et al*. (1990) suggested this may be a result of divergent allele advantage such that alleles from divergent allelic lineages could bind a broader spectrum of antigens and would thus be favored. Alleles from distinct lineages would be preferentially passed on, resulting in the maintenance of divergent lineages in the population persisting over long period of time including speciation events (Lenz 2011) referred to as trans-species polymorphism (Klein *et al*. 1998).

Different antigens interact with specific pathogens so varying patterns of selection among putative loci is anticipated. In Rio Grande silvery minnow, we identified seven positively selected sites (PSS) which were shared between DAB1 and DAB3 alleles. In European chub, three shared PSS were identified between DAB1 and DAB3 alleles (Seifertová & Simková 2011) and more PSS were recognized in DAB3 alleles compared to DAB1 alleles; consistent with our findings and suggestive of stronger positive selection for DAB3 variants. However, this finding does not necessarily imply contemporary selection, as the accumulation of excess non-synonymous substitutions occurs over a species' evolutionary history and it can take a very long time for this signature to be lost in the absence of selection maintaining variation (Garrigan & Hedrick 2003; Bryja *et al*. 2007). Collin *et al*. (2013) also found that DAB1 and DAB3 were driven by different aspects of pathogen-mediated selection. Specifically, selection favored different DAB1 alleles in each population suggesting local adaptation to bacterial pathogens and at DAB3 there was a correlation between genetic diversity and local pathogen diversity. Moreover, genetic differentiation of DAB3 was decoupled from neutral loci and Collin *et al*. (2013) suggested that bacteria (and not parasites) were the main selective agent on DAB3.

Decoupling of neutral and MHC diversity

Several studies have shown that diversity at neutral loci can be decoupled from MHC diversity; suggesting that balancing/fluctuating selection by parasites can maintain diversity at immunologically important genes despite depletion of background genomic diversity (e.g. Tobler *et al*. 2014; van Oosterhout *et al*. 2006; Robertson 1962; Oliver & Piertney 2012; Karvonen & Seehausen 2012). At the level of the individual, MHCIIβ diversity (number of alleles and divergence among alleles) was not correlated with PHt suggesting patterns of diversity at neutral loci and MHC are distinct in Rio Grande silvery minnow; likely a

consequence of different forces acting on these loci. MHCIIβ alleles cannot be assigned to loci in Rio Grande silvery minnow, thus it is not possible to analyze the effects of genetic drift of MHCIIβ allele frequencies but we cannot exclude this effect.

Taking differences in sample size between studies into account, MHC Class IIβ diversity seen in Rio Grande silvery minnow is comparable to other cyprinids including the carp bream where 32 different exon 2 sequences were observed among 20 individuals (Ottova *et al*. 2007) and in European chub where 111 alleles were identified among 15 populations (n=310) (Seifertová *et al*. 2016). Data on North American cyprinid taxa are lacking, with a single study of longnose dace ranging across four river basins (Girard & Angers 2011). This study reported four MHCIIβ alleles across more than 500 individuals and 27 populations. Phylogenetic analysis indicates that these alleles belong to the DAB3 locus so either DAB1 is absent in longnose dace, or the primers used in their study failed to amplify all MHCIIβ loci.

The results presented show that pinpointing key environmental factors that most strongly affect host-parasite interactions is extremely difficult in highly variable systems like the Rio Grande. However, there is substantial evidence that temperature modifies host-parasite interactions (reviewed in Scharsack *et al*. 2016); with increased temperature potentially accelerating growth and lifecycle completion rates of parasites and/or increasing the immune activity of hosts (Brunner & Eizaguirre 2016). Increased temperature can cause an acute stress response in the host (Wegner *et al*. 2008) and can be detrimental to body condition (Dittmer *et al*. 2014). For these reasons it is imperative that diversity is maintained at MHC in Rio Grande silvery minnow so that individuals have an adequate MHC repertoire to respond to pathogens. On a practical level, genetic data presented here provide important baseline information on diversity at adaptively important MHC genes. This will allow future

evaluations of management practices (including captive propagation and augmentation) that could help maintain diversity at these genes in light of persistent low neutral genetic effective size (Osborne *et al*. 2012). Finally, global climate change is predicted to affect aquatic ecosystems in numerous ways. In arid and semi-arid regions, effects include reduced flows and water availability (Milly *et al*. 2005), altered timing of snow-melt runoff and frequency of floods and droughts, and increased water temperatures (Dettinger *et al*. 2015), so developing monitoring programs that integrate genetic and environmental data could help identify drivers of variation at adaptively important genes like MHCIIβ and predict effects of climate change on host-parasite interactions.

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References

Alò D, Turner TF (2005) Effects of habitat fragmentation on effective population size in the endangered Rio Grande silvery minnow. *Conservation Biology* **19(4)**, 1138-1148.

Bessert ML, Ortí G (2003) Microsatellite loci for paternity analysis in the fathead minnow, Pimephales promelas (Teleostei: Cyprinidae). *Molecular Ecology Notes*, 3(4), 532-4.

Bodmer WF (1972) Evolutionary significance of the HL-A system. *Nature*, **237**, 139-83.

Brunner FS, Eizaguirre C (2016). Can environmental change affect host/parasite-mediated speciation? *Zoology*, **119(4)**, 384-394.

Brüniche-Olsen A, Austin JJ, Jones ME *et al*. (2016) Detecting Selection on Temporal and Spatial Scales: A Genomic Time-Series Assessment of Selective Responses to Devil Facial Tumor Disease. *PloS One*, **11(3)**, e0147875.

Brown JL (1997) A theory of mate choice based on heterozygosity. *Behavioral Ecology*, **8(1)**, 60-5.

Bryja J, Charbonnel N, Berthier K *et al*. (2007) Density-related changes in selection pattern for major histocompatibility complex genes in fluctuating populations of voles. *Molecular Ecology*, **16(23)**, 5084-97.

Buonocore F, Randelli E, Casani, D *et al*. (2007) Molecular cloning, differential expression and 3D structural analysis of the MHC class-II β chain from sea bass (*Dicentrarchus labrax* L.). *Fish & shellfish immunology*, **23(4)**, 853-866.

Bush AO, Lafferty KD, Lotz JM, Shostak AW (1997) Parasitology meets ecology on its own terms: Margolis *et al*. revisited. *The Journal of Parasitology*, 575-83.

Chipeta MG, Ngwira BM, Simoonga C, Kazembe LN (2014) Zero adjusted models with applications to analyzing helminths count data. *BMC Research Notes*, **7**, 856

Collin H, Burri R, Comtesse F, Fumagalli L (2013) Combining molecular evolution and environmental genomics to unravel adaptive processes of MHC class IIβ diversity in European minnows (*Phoxinus phoxinus*). *Ecology and Evolution*, **3(8)**, 2568-85.

Consuegra S, de Leaniz CG. (2008) MHC-mediated mate choice increases parasite resistance in salmon. *Proceedings of the Royal Society of London B: Biological Sciences*, **275(1641)**, 1397-403.

Coulon A (2010) genhet: an easy-to-use R function to estimate individual heterozygosity. *Molecular Ecology Resources*, **10(1)**,167-9.

Dettinger M, Udall B, Georgakakos A (2015) Western water and climate change. *Ecological Applications*, **25(8)**, 2069-2093.

Dimsoski P, Toth GP, Bagley MJ (2000) Microsatellite characterization in central stoneroller *Campostoma anomalum* (Pisces: Cyprinidae). *Molecular Ecology*, **9(12)**, 2187-2189.

Dionne M, Miller KM, Dodson JJ, Bernatchez L (2009) MHC standing genetic variation and pathogen resistance in wild Atlantic salmon. *Philosophical Transactions of the Royal Society of London B: Biological Sciences*, **364(1523)**, 1555-1565.

Dionne M, Miller KM, Dodson JJ, *et al*. (2007) Clinal variation in MHC diversity with temperature: evidence for the role of host–pathogen interaction on local adaptation in Atlantic salmon. *Evolution*, **61(9)**, 2154-2164.

Dittmar J, Janssen H, Kuske A *et al*. (2014) Heat and immunity: an experimental heat wave alters immune functions in three-spined sticklebacks (*Gasterosteus aculeatus*). *Journal of Animal Ecology*, **83(4)**, 744-757.

Dixon B, Nagelkerke L, Sibbing F *et al*. (1996) Evolution of MHC Class IIβ chain-encoding genes in the Lake Tana barbel species flock (*Barbus intermedius* complex). *Immunogenetics* B, 419-431.

Doherty PC, Zinkernagel RM (1975) Enhanced immunological surveillance in mice heterozygous at the H-2 gene complex. *Nature*, **256**, 50 – 52.

Dudley RK, Platania SP, White GC (2014) Rio Grande Silvery Minnow Population Monitoring Program results from May to December. Report submitted to the U.S. Bureau of Reclamation Albuquerque Office. 151 pp.

Dudley RK, Helfrich DA, Platania SP (2009) Effects of river intermittency on populations of Rio Grande silvery minnow. Report submitted to the U.S. Bureau of Reclamation Albuquerque Office. 63 pp.

Eizaguirre C, Lentz TL (2010) Major histocompatibility complex polymorphism: dynamics and consequences of parasite-mediated local adaptation in fishes. *Journal of Fish Biology*, **77(9)**, 2023-47.

Eizaguirre C, Lenz TL, Kalbe M, Milinski M (2012) Rapid and adaptive evolution of MHC genes under parasite selection in experimental vertebrate populations. *Nature Communications*, **3**, 621.

Ellis AE (2001) Innate host defense mechanisms of fish against viruses and bacteria. *Developmental & Comparative Immunology*, **25(8)**, 827-839.

Garrigan D, Hedrick PW (2003) Perspective: detecting adaptive molecular polymorphism: lessons from the MHC. *Evolution*, **57(8)**, 1707-22.

Girard P, Angers B (2011) The functional gene diversity in natural populations over postglacial areas: the shaping mechanisms behind genetic composition of longnose dace (*Rhinichthys cataractae*) in northeastern North America. *Journal of Molecular Evolution*, **73(1-2)**,45-57.

Goudet J (1995) FSTAT (Version 1.2): A computer program to calculate F-statistics. *Journal of Heredity*, **86**, 485-486.

Hanley JA, Negassa A, Forrester JE (2003) Statistical analysis of correlated data using generalized estimating equations: an orientation. *American Journal of Epidemiology*, **157**, 364-375.

Hansson B, Westerberg L (2002) On the correlation between heterozygosity and fitness in natural populations. *Molecular Ecology,* **11(12)**, 2467-74.

Hedrick PW, Thomson G, Klitz W (1987) Evolutionary genetics and HLA: another classic example. *Biological Journal of the Linnean Society*, **31(4)**, 311-331.

Hedrick PW (2002) Pathogen resistance and genetic variation at MHC loci. *Evolution*, **56(10)**, 1902-8.

Hill AVS (1991) HLA associations with malaria in Africa: some implications for MHC evolution. In *Molecular evolution of the major histocompatibility complex* (eds Klein J, Klein D), pp. 403–419. Berlin, Germany: Springer.

Hillis DM, Moritz C, Mable BK eds, (1996) *Molecular Systematics*. Sunderland, MA: Sinauer Associates.

Højsgaard S, Halekoh U, Yan J (2006) The R Package geepack for Generalized Estimating Equations. *Journal of Statistical Software*, **15**, 1 – 11.

Jackman S (2015) pscl: Classes and Methods for R Developed in the Political Science Computational Laboratory, Stanford University. Department of Political Science, Stanford University. Stanford, California. R package version 1.4.9. URL http://pscl.stanford.edu/.

Jelks HL, Walsh SJ, Burkhead NM, *et al*. (2008) Conservation status of imperiled North American freshwater and diadromous fishes. *Fisheries*, **33(8)**, 372-407.

Jones SR, Palmen M, van Muiswinkel WB. (1993) Effects of inoculum route and dose on the immune response of common carp, *Cyprinus carpio* to the blood parasite*, Trypanoplasma borreli*. *Veterinary Immunology and Immunopathology*, **36(4)**, 369-78.

Jones SR, Woo PT (1987) The immune response of rainbow trout, *Salmo gairdneri* Richardson, to the haemoflagellate, *Cryptobia salmositica* Katz, 1951. *Journal of Fish Diseases*, **10(5)**, 395-402.

Jones SR (2001) The occurrence and mechanisms of innate immunity against parasites in fish. *Developmental & Comparative Immunology*, **25(8)**, 841-52.

Kalbe M, Wegner KM, Reusch TB (2002) Dispersion patterns of parasites in 0+ year three-spined sticklebacks: a cross population comparison. *Journal of Fish Biology*, **60(6)**, 1529-42.

Karvonen A, Rintamäki P, Jokela J, Valtonen ET (2010) Increasing water temperature and disease risks in aquatic systems: climate change increases the risk of some, but not all, diseases. *International Journal for Parasitology*, **40(13)**, 1483-8.

Karvonen A, Seehausen O (2012) The role of parasitism in adaptive radiations—when might parasites promote and when might they constrain ecological speciation? *International Journal of Ecology*, **2012**, 1-20.

Klein J (1986) *The natural history of the major histocompatibility complex.* New York, NY: John Wiley.

Klein J, O'Huigin C (1994) MHC polymorphism and parasites. *Philosophical Transactions of the Royal Society of London Series B, Biological Sciences*, **346**, 351–358.

Kurtz J, Wegner KM, Kalbe M *et al*. (2006) MHC genes and oxidative stress in sticklebacks: an immuno-ecological approach. *Proceedings of the Royal Society of London B: Biological Sciences*, **273(1592)**, 407-14.

Lafferty KD, Holt RD (2003) How should environmental stress affect the population dynamics of disease? *Ecology Letters*, **6(7)**, 654-664.

Landry C, Bernatchez L (2001) Comparative analysis of population structure across environments and geographical scales at major histocompatibility complex and microsatellite loci in Atlantic salmon (*Salmo salar*) *Molecular Ecology*, **10**, 2525–2539.

Lenz TL (2011) Computational prediction of MHC II-antigen binding supports divergent allele advantage and explains trans-species polymorphism. *Evolution*, **65(8)**, 2380-90.

Lõhmus M, Björklund M (2015) Climate change: what will it do to fish–parasite interactions? *Biological Journal of the Linnean Society*, **116(2)**, 397-411.

MacDougall-Shackleton EA, Derryberry EP, Foufopoulos J *et al*. (2005) Parasite-mediated heterozygote advantage in an outbred songbird population. *Biology Letters*, **1(1)**, 105-7.

MacNab V, Barber I (2012) Some (worms) like it hot: fish parasites grow faster in warmer water, and alter host thermal preferences. *Global Change Biology*, **18(5)**, 1540-1548.

Marcogliese DJ, Locke SA, Gélinas M, Gendron AD (2016) Variation in parasite communities in spottail shiners (*Notropis hudsonius*) linked with precipitation. *The Journal of Parasitology*, **102(1)**, 27-36.

Meyer D, Thomson G (2001) How selection shapes variation of the human major histocompatibility complex: a review. *Annals of Human Genetics*, **65(1)**, 1-26.

Miller MA, Pfeiffer W, Schwartz T (2010) Creating the CIPRES Science Gateway for inference of large phylogenetic trees. In: Gateway Computing Environments Workshop (GCE), 2010 Nov 14 (pp. 1-8). IEEE.

Milly PC, Dunne KA, Vecchia AV (2005) Global pattern of trends in streamflow and water availability in a changing climate. *Nature*, **438** (7066), 347-350.

Nelson JS (2006) Fishes of the World. 4th ed. Hoboken, New Jersey, USA: John Wiley & Sons. xix+601 pp.

Noga EN (2000) Fish diseases (diagnosis and treatment). Blackwell Science Ltd. USA 378 pp.

Oksanen J (2015) Vegan: an introduction to ordination. URL http://cran. r-project. org/web/packages/vegan/vignettes/introvegan.

Oliver MK, Lambin X, Cornulier T, Piertney SB (2009) Spatio-temporal variation in the strength and mode of selection acting on major histocompatibility complex diversity in water vole (*Arvicola terrestris*) metapopulations. *Molecular Ecology*, **18(1)**, 80-92.

Oliver MK, Piertney SB (2012) Selection maintains MHC diversity through a natural population bottleneck. *Molecular Biology and Evolution*, **29(7)**, 1713-20.

Ono H, Klein D, Vincek V *et al*. (1992) Major Histocompatibility Complex Class II genes in zebrafish. *Proceedings National Academy of Sciences USA*. **89**, 11886-11890.

Osborne MJ, Carson EW, Turner TF (2012) Genetic monitoring and complex population dynamics: insights from a 12-year study of the Rio Grande silvery minnow. *Evolutionary Applications*, **5(6)**, 553-574.

Osborne MJ, Turner TF (2011) Isolation and characterization of major histocompatibility class IIβ genes in an endangered North American cyprinid fish, the Rio Grande silvery minnow (*Hybognathus amarus*). *Fish and Shellfish Immunology*, **30(6)**, 1275-1282.

Ottova E, Simkova A, Morand S (2007) The role of major histocompatibility complex diversity in vigour of male fish (*Abramis brama* L.) and parasite selection. *Biological Journal of the Linnean Society*, **90**, 525-538.

Petit RJ, El Mousadik A, Pons O (1998) Identifying populations for conservation on the basis of genetic markers. *Conservation Biology*, **12**, 844-855.

Posada D (2009) Selection of Models of DNA Evolution with jModelTest. *Bioinformatics for DNA sequence analysis*, 93-112.

Rakus KŁ, Irnazarow I, Forlenza M *et al*. (2009). Classical crosses of common carp (*Cyprinus carpio* L.) show co-segregation of antibody response with major histocompatibility class IIβ genes. *Fish & Shellfish Immunology*, **26(3)**, 352-8.

Reed DH, Frankham R (2003) Correlation between fitness and genetic diversity. *Conservation Biology*, **17**, 230–237.

Rice WR. (1989) Analyzing tables of statistical tests. *Evolution*, **43(1)**, 223-5.

Robertson A (1962) Selection for heterozygotes in small populations. *Genetics*, **47**, 1291-1300.

Ronquist F, Huelsenbeck JP (2003) MrBayes 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics*, **19(12)**, 1572-1574.

Saccheri I, Kuussaari M, Kankare M *et al*. (1998) Inbreeding and extinction in a butterfly metapopulation. *Nature*, **392**, 491–494.

Sasal P, Trouvé S, Müller-Graf C, Morand S (1999) Specificity and host predictability: a comparative analysis among monogenean parasites of fish. *Journal of Animal Ecology*, **68(3)**, 437-444.

Savage AE, Zamudio KR (2016) Adaptive tolerance to a pathogenic fungus drives major histocompatibility complex evolution in natural amphibian populations. *Proceedings of the Royal Society of London B*, **283(1827)**, 20153115.

Scharsack JP, Franke F, Erin NI *et al*. (2016) Effects of environmental variation on host–parasite interaction in three-spined sticklebacks (*Gasterosteus aculeatus*). *Zoology*, **119(4)**, 375-383.

Seifertová M, Šimková A (2011) Structure, diversity and evolutionary patterns of expressed MHC class IIβ genes in chub (*Squalius cephalus*), a cyprinid fish species from Europe. *Immunogenetics*, **63(3)**, 167-81.

Seifertová M, Jarkovský J, Šimková A (2016) Does the parasite-mediated selection drive the MHC class IIβ diversity in wild populations of European chub (*Squalius cephalus*)? *Parasitology Research*, **115(4)**, 1401-15.

Spurgin LG, Richardson DS. (2010) How pathogens drive genetic diversity: MHC, mechanisms and misunderstandings. *Proceedings of the Royal Society of London B,* **277**, 979–988.

Tamura K, Peterson D, Peterson N *et al*. (2010) MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Molecular Biology and Evolution*, **28(10)**, 2731-9.

Tobler M, Plath M, Riesch R *et al*. (2014) Selection from parasites favors immunogenetic diversity but not divergence among locally adapted host populations. *Journal of Evolutionary Biology*, **27(5)**, 960-74.

Turner TF, Dowling TE, Broughton RE, Gold JR. Variable microsatellite markers amplify across divergent lineages of cyprinid fishes (subfamily Leusicinae). *Conservation Genetics*, 5(2), 279-81.

Turner TF, Osborne MJ, Moyer G *et al*. (2006) Life history and environmental variation interact to determine effective population to census size ratio. *Proceedings of the Royal Society of London B: Biological Sciences*, **273(1605)**, 3065-3073.

U.S. Department of the Interior (1994) Endangered and threatened wildlife and plants: final rule to list the Rio Grande silvery minnow as an endangered species. *Federal Register*, **59**, 36988–36995.

Van Erp SHM, Egberts E, Stet RJM (1996) Characterization of major histocompatibility complex class IIA and B genes in a gynogenetic carp clone. *Immunogenetics*, **44**, 192–202.

Van Oosterhout C, Joyce DA, Cummings SM, *et al*. (2006) Balancing selection, random genetic drift, and genetic variation at the major histocompatibility complex in two wild populations of guppies (*Poecilia reticulata)*. *Evolution*, **60(12)**, 2562-74.

Wakeland EK, Boehme S, She JX *et al*. (1990) Ancestral polymorphisms of MHC class II

genes: divergent allele advantage. *Immunologic Research,* **9(2)**, 115-122.

Wegner KM, Kalbe M, Milinski M, Reusch TB (2008) Mortality selection during the 2003 European heat wave in three-spined sticklebacks: effects of parasites and MHC genotype. *BMC Evolutionary Biology*, **8(1)**, 124.

Wegner KM, Kalbe M, Rauch G *et al*. (2006) Genetic variation in MHC class II expression and interactions with MHC sequence polymorphism in three-spined sticklebacks. *Molecular Ecology*, **15(4)**, 1153-64.

Wegner KM, Reusch TB, Kalbe M (2003) Multiple parasites are driving major histocompatibility complex polymorphism in the wild. *Journal of Evolutionary Biol*ogy, **16,** 224-232.

Westemeier RL, Brawn JD, Simpson SA *et al*. (1998) Tracking the long-term decline and recovery of an isolated population. *Science*, **282**, 1695–1698.

Whyte SK, Allan JC, Secombes CJ, Chappell LH (1987) and diplostomules of *Diplostomum spathaceum* (Digenea) elicit an immune response in rainbow trout, *Salmo gairdneri* Richardson. *Journal of Fish Biology*, **31**,185-90.

Yang Z (1997) PAML: a program package for phylogenetic analysis by maximum likelihood *Computer Applications in BioSciences*, **13**, 555-556.

Zhang YX, Chen SL, Liu YG *et al*. (2006) Major histocompatibility complex class IIB allele polymorphism and its association with resistance/susceptibility to *Vibrio anguillarum* in Japanese flounder (*Paralichthys olivaceus*). *Marine Biotechnology*, **8(6)**, 600-610.

Zuur AF, Ieno EN, Walker NJ, *et al*. (2009) Mixed effects models and extensions in ecology with R. New York: Springer.

Data Accessibility

DNA sequence data will be made accessible on GenBank. Data used in this study are accessible on Dryad doi:10.5061/dryad.bp7s3

Author Contributions

M.J.O, J.L contributed to study design, sample collection and data analysis. T.J.P conducted statistical analysis and prepared figures. All authors contributed to writing the manuscript.

Figure 1. Map of the study sites (labeled 1-6) in the Rio Grande, New Mexico, USA.

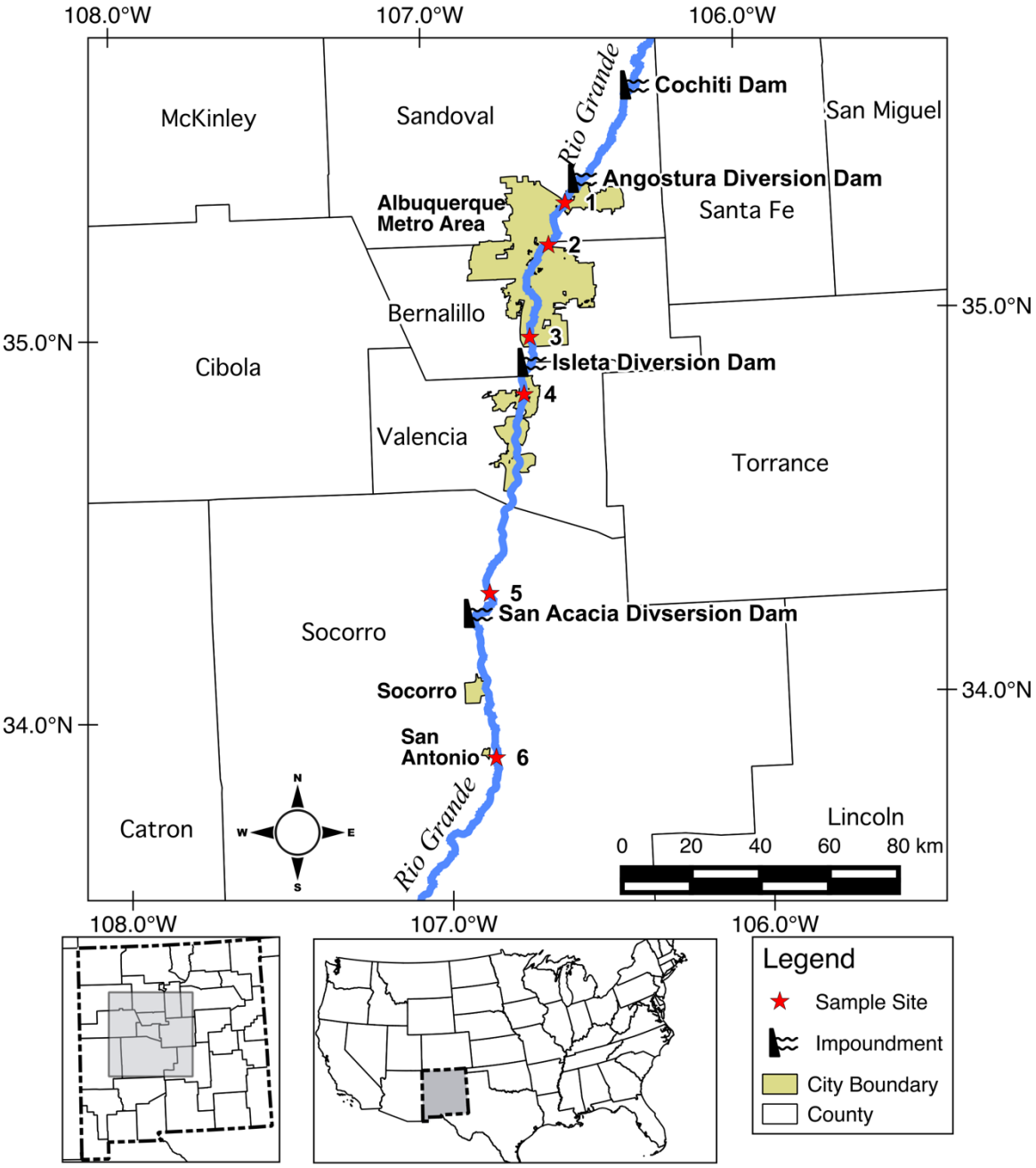

Figure 2. Variation in Rio Grande silvery minnow parasite infracommunities across sites in the Rio Grande and between years (2006-black symbols, 2008- gray symbols). Infracommunity resemblance was quantified using Bray-Curtis dissimilarity of square root transformed abundance of the four most abundant parasite taxa and analysed using nonmetric multidimensional scaling (stress = 0.022). Sites correspond to longitudinal position in Rio Grande, 1 being most upstream and 6 the most downstream site (see Figure 1).

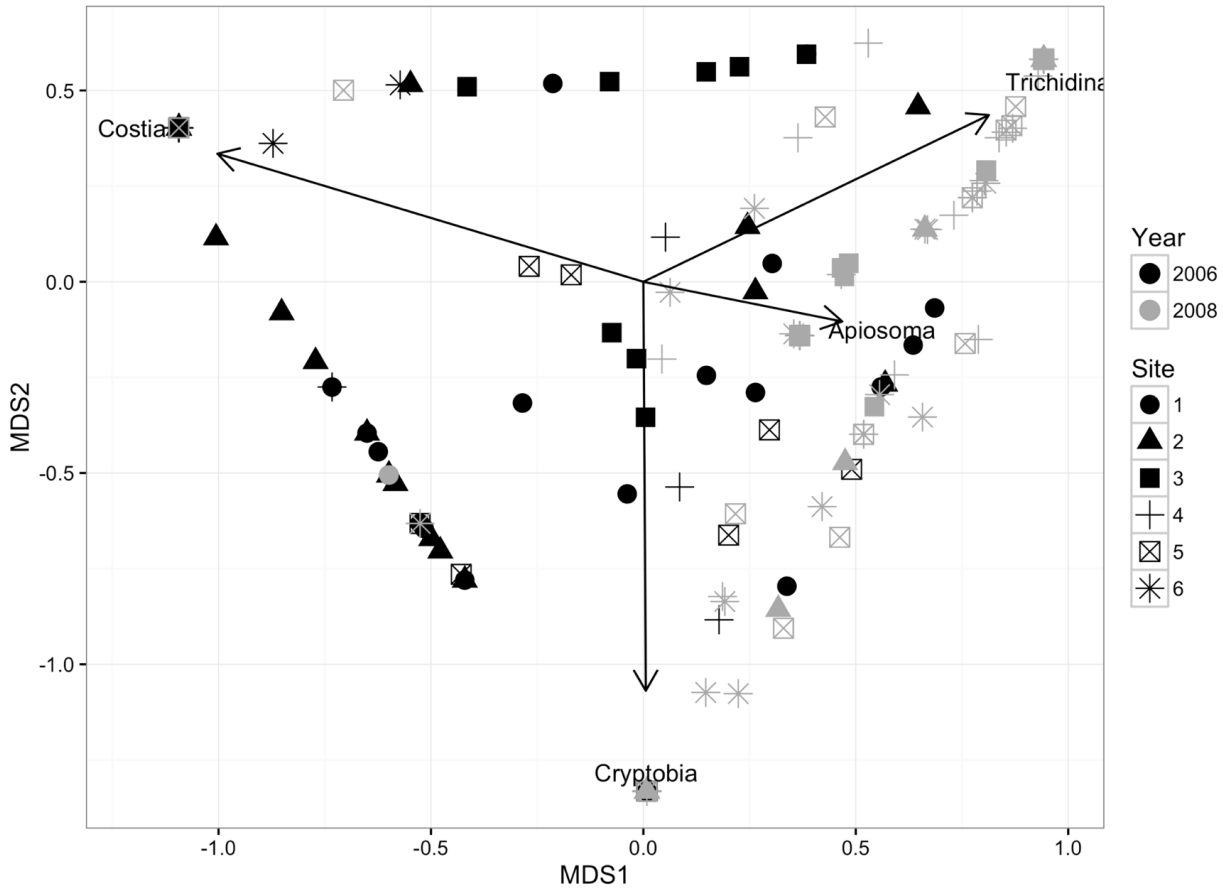

Figure 3. Site and year differences in Rio Grande silvery minnow parasite load (individual parasitization index), and infracommunity diversity (Shannon-Wiener) and evenness. Drawn are mean estimate (+- standard errors) for each site and year (2006-black bars, 2008-gray bars). Sites correspond to longitudinal position in Rio Grande, 1 being most upstream and 6 the most downstream site (see Figure 1).

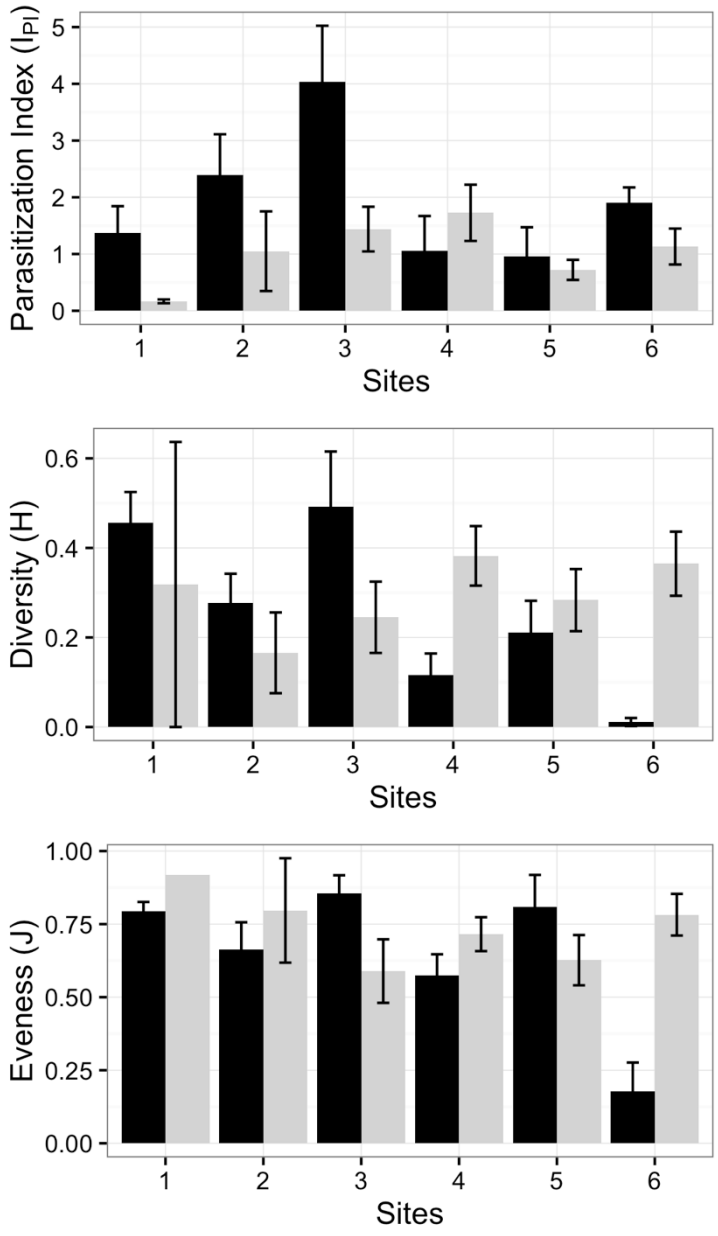

Table 1. Environmental and parasite data by year and site (1. Bernalillo, 2. Alameda, 3. Los Padillas, 4. Los Lunas, 5. La Joya, 6. San Antonio). Environmental parameters include mean daily discharge (cubic meters per second), water temperature (degrees Celsius [C]), and water pH. Also shown are mean body size (mm) of Rio Grande silvery minnow. Parasite data includes number of individuals assessed for parasites (N), proportion of infected individuals, mean parasitization index (I_{PI}), Shannon diversity (H) and evenness (J), mean number of parasites on the infracommunity, mean, minimum and maximum number of parasite taxa per site.

Site	**1**		**2**		**3**		**4**		**5**		**6**	
Year	**2006**	**2008**	**2006**	**2008**	**2006**	**2008**	**2006**	**2008**	**2006**	**2008**	**2006**	**2008**
Daily Discharge (m^3 per sec^{-1})	77.47	72.02	13.78	26.29	16.57	26.25	1.49	27.82	0.76	12.20	1.06	19.23
Water Temperature (°C)	23.45	19.76	23.48	22.22	23.75	22.68	24.69	21.35	26.46	22.08	24.52	22.91
pH	8.15	8.24	7.38	8.33	7.86	7.96	8.20	8.09	7.95	8.28	9.36	8.31
DO (mgL)	6.21	7.37	7.25	7.01	NA	7.21	7.29	6.76	6.08	6.95	7.02	6.99
Fish body size (mm)	54.33	52.87	55.20	50.20	62.08	19.57	53.20	27.80	56.15	19.10	45.95	27.10
Parasite Community												
n	30	30	30	30	13	14	30	30	30	30	30	29
Proportion Infected	0.857	0.10	0.95	0.333	0.846	0.714	0.65	0.967	0.800	0.833	0.650	1.000
Mean I_{PI}	1.372	0.167	2.396	1.050	4.036	1.440	1.059	1.726	0.958	0.721	1.900	1.132
Mean H	0.457	0.318	0.278	0.166	0.492	0.245	0.116	0.382	0.211	0.283	0.011	0.365
Mean Number Parasites of Infracommunity	26.483	0.778	45.767	5.200	72.714	11.928	9.567	17.933	41.700	10.448	23.667	12.588
Mean Number Taxa	1.667	0.133	1.600	0.400	1.692	1.214	0.900	1.900	1.100	1.800	0.700	1.966
Min Taxa	0	0	0	0	0	0	0	0	0	0	0	1
Max Taxa	3	2	3	2	3	3	2	5	3	5	2	4

Table 2. Results of PERMANOVA on Bray-Curtis dissimilarities of parasite infracommunities on Rio Grande silvery minnow sampled from six locations in the Rio Grande for (A) all years combined, (B) during 2006 and (C) 2008.

Source	df	SS	MS	Pseudo-*F*	*P*	Estimated Component of variation
A) All Years						
Body size	1	9.515	9.515	43.781	0.001	0.134
Site	5	5.187	1.037	4.773	0.001	0.073
Year	1	5.029	5.029	23.137	0.001	0.071
Site x Year	5	5.538	1.108	5.096	0.001	0.078
Residuals	210	45.642	0.218	-	-	0.644
Total	222	70.911	-	-	-	1
B) 2006						
Body size	1	0.387	0.387	1.826	0.116	0.011
Site	5	9.067	1.813	8.555	0.001	0.261
Residuals	119	25.223	0.212	-	-	0.726
Total	125	34.676	-	-	-	1
C) 2008						
Body size	1	1.004	1.004	4.513	0.007	0.044
Site	5	1.708	0.342	1.536	0.080	0.075
Residuals	90	20.014	0.222	-	-	0.881
Total	96	22.726	-	-	-	1

Table 3. Genetic diversity statistics by site (1. Bernalillo, 2. Alameda, 3. Los Padillas, 4. Los Lunas, 5. La Joya, 6. San Antonio) and year for microsatellites (sample size [n], gene diversity [H_e], observed heterozygosity [H_o]), mean number of alleles [MNA], allelic richness [A_R], individual heterozygosity [PHt], average inbreeding coefficient [F_{IS}]), and MHCIIβ including sample size [n], nucleotide diversity [π], number of synonymous (d_S) and nonsynonymous (d_N) substitutions between alleles, allelic richness (N_{ac}), mean number of alleles (MNA), total number of MHCIIβ alleles, number of MHC DAB1 and DAB3 alleles ($N_{a(DAB1/DAB3)}$), average amino acid p-distance among alleles (within individuals) and maximum number of MHCIIβ alleles per individual.

	Site	**1**		**2**		**3**		**4**		**5**		**6**	
	Year	**2006**	**2008**	**2006**	**2008**	**2006**	**2008**	**2006**	**2008**	**2006**	**2008**	**2006**	**2008**
Microsatellites	n	30	30	30	30	13	14	30	30	30	30	30	29
	H_e	0.839	0.849	0.830	0.867	0.823	0.843	0.855	0.844	0.833	0.842	0.829	0.838
	H_O	0.731	0.720	0.697	0.684	0.743	0.620	0.713	0.697	0.705	0.701	0.699	0.717
	MNA	13.111	14.000	13.778	14.667	9.444	10.111	13.667	13.667	14.000	13.000	12.778	13.440
	$A_{R(n=12)}$	9.848	9.974	9.664	10.299	9.209	9.596	9.994	9.868	9.908	9.250	9.322	9.622
	PHt	0.714	0.705	0.646	0.671	0.739	0.616	0.714	0.697	0.703	0.685	0.701	0.699
	F_{IS}	0.131	0.155	0.163	0.214	0.101	0.261	0.168	0.177	0.156	0.194	0.167	0.150
MHCIIβ	n	21	26	19	25	13	12	20	25	18	28	20	25
	Π	0.103	0.158	0.104	0.092	0.104	0.21	0.108	0.21	0.14	0.158	0.087	0.171
	d_S	4.166	6.49	4.488	2.159	4.787	6.464	4.744	5.236	7.971	6.251	1.944	7.04
	d_N	27.261	37.162	29.112	22.245	28.941	36.945	30.835	32.701	41.393	36.066	21.13	39.07
	N_{ac}	8.657	8.725	8.579	8.938	7.694	9.646	8.566	8.597	6.168	8.704	7.716	8.740
	MNA	2.095	1.467	2.050	1.267	1.538	1.929	2.150	1.600	1.550	1.500	1.800	1.172
	Total Alleles	24	22	21	23	14	21	21	25	12	24	20	21

$N_{a(DAB1/DAB3)}$	22/2	18/4	19/2	21/2	11/3	18/3	19/2	22/3	10/2	20/4	17/3	16/5
Average amino acid p-distance	0.190	0.135	0.139	0.079	0.125	0.185	0.183	0.153	0.159	0.141	0.152	0.152
Max Alleles	3	3	4	4	2	4	4	3	3	3	3	2

Table 4. Tests of selection on MHCIIβ for DAB1 and DAB3 alleles and across all alleles (DAB1 and DAB3). Positively selected codons shared by DAB1 and DAB3 are indicated in bold. Single letter amino acid codes are given after the codon position number.

Duplicate	Model	Ln L	Positively selected sites (codon position and amino acid)	LRT statistic
DAB1	M1a	-1929.52	Not Allowed	
	M2a	-1841.927	**7R, 9T**, 13H, 21I, **23F**, 34Y, 56E, 57L, **58W**, **64I**, 69R, **71Q**, **74Y**, 77Y, 81L, 82Y, 84A	175.185 *P*<0.001 df=2
	M7	-1931.606	Not Allowed	
	M8	- 1841.965	Log(theta) **7R, 9T**, 13H, 21I, **23F**, 34Y, 56E, 57L, **58W**, **64I**, 69R, **71Q**, **74Y**, 77Y, 81L, 82Y, 84A	179.281 *P*<0.001 df=2
DAB3	M1a	-697.848	Not Allowed	
	M2a	-681.473	5L, **7I**, **9N**, 12I, **23F**, 25Q, 37F, 50F, 54Q, **58L** **64R**, 66L, 68L, **71E**, 75L, 83F, 87V	32.749 *P*<0.001 df=2
	M7	-697.877	Not Allowed	
	M8	-681.474	5L, **7I**, **9N**, 12I, **23F**, 25Q, 37F, 50F, 54Q, **58L** **64R**, 66L, 68L, **71E**, 75L, 83F, 87V	32.806 *P*<0.001 df=2
DAB1 & DAB3	M1a	-2902.369	Not Allowed	
	M2a	-2743.214	7R, 9T, 34Y, 57L, 58W, 71Q, 74Y,81L, 82Y, 84A	318.310 *P*<0.001 df=2
	M7	-2906.390	Not Allowed	
	M8	-2747.396	7R, 9T, 23F, 34Y, 43K, 50H, 56E, 57L, 58W, 64I, 71Q, 74Y, 77Y, 81L, 82Y, 84A	317.989 *P*<0.001 df=2

Table 5. ZINB count models identifying associations between abundance of a particular parasite taxa and presences/absence of MHC alleles in Rio Grande silvery minnow. Bolded values indicate alleles that were significant at $P < 0.05$ and marginally so at $P < 0.1$ ([a]).

Parasite	Coefficient	Estimate	Std. Error	P-value
Ichthyobodo	(Intercept)	2.767	0.433	< 0.001
	DAB1*03	-0.498	0.583	0.393
	DAB1a*06	-0.256	0.705	0.716
	DAB1*13	1.089	0.797	0.172
	DAB1*05	-1.207	1.194	0.312
	DAB1a*04	**-2.568**	**0.898**	**0.004**
	DAB3*06	-2.513	1.730	0.146
	DAB1*07	1.310	1.143	0.252
	DAB1*08	0.762	0.919	0.407
	DAB1*12	**-2.104**	**1.041**	**0.043**
	DAB1a*02	-1.005	0.942	0.286
	DAB1*04	**3.693**	**1.663**	**0.026**
	Log(theta)	-1.889	0.340	< 0.001
Trichodina	(Intercept)	1.941	0.245	<0.001
	DAB1*03	0.507	0.574	0.378
	DAB1a*06	-0.018	0.593	0.976
	DAB1*13	0.045	0.513	0.930
	DAB1*05	-0.514	0.554	0.354
	DAB1a*04	0.250	0.622	0.688
	DAB3*06	0.333	0.518	0.521
	DAB1*07	0.218	0.632	0.730
	DAB1*08	0.165	0.760	0.828
	DAB1*12	0.537	0.687	0.434
	DAB1a*02	**-2.262**	**0.885**	**0.011**
	DAB1*04	0.028	0.704	0.969
	Log(theta)	-0.704	0.284	0.013
Cryptobia	(Intercept)	-0.379	0.309	0.220
	DAB1*03	0.285	0.603	0.636
	DAB1a*06	**1.216**	**0.715**	**0.089[a]**
	DAB1*13	**2.239**	**0.782**	**0.004**
	DAB1a*04	0.580	0.824	0.482

	DAB1*07	**2.315**	**0.860**	**0.007**
	DAB1*12	**1.611**	**0.871**	**0.064**[a]
	DAB1a*02	0.169	0.929	0.855
	Log(theta)	-2.342	0.166	<0.001
Apiosoma	(Intercept)	-0.448	0.286	0.117
	DAB1a*06	1.164	0.718	0.105
	DAB1*13	**1.698**	**0.738**	**0.021**
	DAB1*08	0.663	0.873	0.448
	DAB1*12	**2.446**	**0.876**	**0.005**
	DAB1a*02	1.434	0.884	0.105

Table 6. Results of general estimating equations used to evaluate the relationship between parasite infections (measured as infracommunity richness, total abundance, and index of parasitization [I_{PI}]) and genetic diversity at neutral microsatellite loci (PHt), number of MHCIIβ alleles (MHC A), and maximum amino acid p-distance between DAB alleles within an individual (AA Dist).

Response variable	**Predictor**	**Estimate**	**S.E.**	**Pr(>|W|)**
Richness	(Intercept)	-3.383	0.344	<0.001
	MHC A	-0.062	0.095	0.516
	PHt	-0.502	0.358	0.161
	AA Dist	-0.200	0.369	0.588
Abundance	(Intercept)	-1.136	0.619	0.066
	PHt	-1.244	0.564	**0.028**
	AA Dist	-0.649	0.352	0.065
I_{PI}	(Intercept)	-2.856	0.286	<0.001
	PHt	-0.636	0.305	**0.037**
	AA Dist	-0.226	0.109	**0.038**