Collaborative Research and Monitoring: Evaluation of the Rio Grande Silvery Minnow Health in Relation to Changes in Water Quality, Pathogens and Other Environmental Stressors:

Multi-locus Major Histocompatibility Complex Class IIβ and Parasite Diversity in the Rio Grande Silvery Minnow.

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Executive Summary

Long-term persistence of species depends partially on avoiding loss of genetic variation (Hedrick *et al.* 1996). This variation forms the basis of a species' ability to adapt and respond to changing environments. For example, genetic variation of genes involved with immunity serves as the foundation of an individual's response to disease pressures. Variation at immune genes is especially important for aquatic species, whose chemical and microbial environment is impacted heavily by humans, which may increase their risk of exposure to pathogens (De Swart et al. 1996; Harvell et al. 1999). In this portion of the fish health study we characterized and measured diversity at genes of the major histocompatibility complex (MHC) Class IIβ in the Rio Grande silvery minnow, *Hybognathus amarus*. These data were used to examine the relationship between MHC variation and pathogen diversity. Major findings of this study were:

- Seventy-two different MHC alleles were identified based on MHC Class IIβ Exon two cDNA sequences and all alleles shared conserved features of classical MHC Class IIβ.

- Three divergent groups of alleles were identified on the basis of phylogenetic analysis.

- The expression of Hyam-DAB1*05 was associated with lighter *Costia* spp. infection whilst expression of Hyam-DAB2*06 was associated with heavier *Costia* spp. infection. Expression of Hyam-DAB1*13 was associated with heavier *Apiosoma* infection. No relationship was detected between presence or absence of Hyam-DAB1*03 or Hyam-DAB2*04 an infection with particular species.

- No association between gill parasite diversity (number of species per individual) or abundance and MHC diversity (number of MHC alleles per fish) was detected.

- No association was detected between pathogen diversity or abundance and the number of MHC allelic groups expressed by individuals.

- Parasite and bacterial species diversity and abundance also differed spatially.

Introduction

It is widely recognized that there is a relationship between levels of genetic diversity and a species' ability to respond to changing environmental conditions such as exposure to novel pathogens (e.g. Reed and Frankham 2003) and that there can be a negative relationship between the probability of extinction and the amount of genetic variation (Saccheri et al. 1998; Westemeier et al. 1998). In small populations, extinction risk may be heightened if individuals lack the diversity necessary to face infections from metazoan parasitic, bacterial and viral infections. In degraded environments, particularly aquatic ones, endangered species may be confined to areas (such as isolated pools) where they are more susceptible to infection. A case in point is the Rio Grande silvery minnow, a federally endangered North American cyprinid that now occupies a a fraction of its historic range in the Rio Grande, New Mexico. The Rio Grande is a modified and highly impacted river system. From spring to fall, river flows are largely diverted to supply agricultural lands and to meet interstate water delivery obligations. This can cause parts of the Rio Grande to be intermittent and, as a consequence, fish can become confined to pools. Under these conditions, fish are exposed to degraded water quality (e.g. increased temperatures and decreased dissolved oxygen) and increased competition and predation. High fish densities can increase the probability of exposure to pathogens and exacerbate susceptibility to infection due to stress. To date we have tracked overall measures of genetic diversity in Rio Grande silvery minnow including allelic richness, heterozygosity and genetic effective size (Alò & Turner 2005; Turner et al. 2006; Turner & Osborne 2006). The markers used to track these parameters are presumably neutral (i.e. not under selection). Although these neutral markers provide crucial information about demography, genetic effective size, and stocking practices, they do not allow us to make specific predictions about whether the species maintains the genetic diversity needed to respond to disease pressures. This study aims to address these concerns by examining genes of the major histocompatibility complex (MHC), a critical component of the adaptive immune response in vertebrates (e.g. Klein 1986).

Major histocompatibility complex genes encode receptor molecules that recognize and bind foreign peptides, which are then presented to immune cells, initiating an immune response. MHC comprises two subgroups that are involved with immune function: i) Class I molecules that are expressed on the surface of nucleated cells and

are responsible for defense against intracellular pathogens including viruses; and ii) Class II molecules that occur on antigen-presenting cells and are involved with defense against extra-cellular pathogens such as bacteria, arthropods and helminths. Nucleotide sequence variation within the peptide binding region of MHC is directly related to the individual's ability to mount an immune response to specific suites of pathogens (e.g. Davenport & Hill 1996; Hill 2001). Hence, MHC is highly polymorphic, containing the most variable genes described in vertebrates. This variation may also be a product of the role that MHC appears to play in mate choice in some species (e.g. Landry & Bernatchez 2001; Milinski. 2006; Penn & Potts 1999). One of the most important distinctions between the MHC genes in bony fish and other vertebrates, is that the two classes of MHC genes are found on separate chromosomes in bony fish (Bingulac-Popvic et al. 1997; Sato et al. 2000).

Diversity at MHC is thought to be maintained by over-dominant or frequency-dependent selection (forms of balancing selection). Over-dominant selection describes the case whereby the heterozygous condition at a locus is advantageous. In the case of MHC, having multiple alleles at a locus may allow an immune response to be mounted against different suites of pathogens. Frequency-dependent selection is a dynamic process whereby the relative fitness of an allele varies depending upon its frequency within the population. Variation can also be maintained by diversifying selection, whereby genotypes that depart from the average condition in either direction are favored. In some fish, like brown trout, particular MHC alleles may confer resistance to particular pathogens (e.g. Langefors et al. 2001; Paterson et al. 1998). There are also cases where an MHC allele causes susceptibility to a disease (reviewed in Apanius et al. 1997). In other fish, overall heterozygosity at MHC is related to survivorship from infection (Hedrick et al. 2001a; Arkush et al. 2002, Wegner et al. 2003). There is also evidence that parasites drive MHC variability; however, evidence also suggests that there is an optimal number of MHC alleles within individuals (e.g. Wegner et al. 2003).

MHC sequences have been characterized to varying degrees in bony fishes. These include various trout and salmon, pufferfish, sticklebacks, cichlids and several representatives of the fish family Cyprinidae including common carp (*Cyprinus carpio*) (Ono *et al.* 1993; van Erp *et al.* 1996), zebrafish (*Danio rerio*) (Ono *et al.* 1992, Sültmann *et al.* 1993; Sültmann *et al.* 1994) and barbs (*Barbus intermedius* complex) (Dixon *et al.*

1996; Kruiswijk *et al.* 2004). More recently, complementary DNA[1] (cDNA) sequences have been obtained from additional cyprinids including several representatives of the subfamilies Cypininae, Gobioninae, Tincinae and Leucisinae (Ottova *et al.* 2005). These studies have provided crucial insight into the organization of the MHC regions in the Cyprinidae, the largest family of freshwater fishes in the world (~2400 species). A major finding of these studies was the identification of two groups of divergent alleles (DAB1 and DAB3) but it is not clear how many loci these represent.

Although there are around 270 endemic North America cyprinids, there have been no descriptions or published studies of their MHC genes. Despite the large number of species worldwide, fewer than five population-level studies have been published that considered MHC genes within this group of fishes. Population studies have been conducted in the Lake Tana African 'large' barb (*Barbus intermedius*) species flock (Kruiswijk et al. 2005) and in carp bream (*Abramis brama*) (Ottova et al. 2007). The latter study is the first (to our knowledge) that simultaneously considered the correlation between diversity at two transcribed MHC allelic groups and parasite load, parasite diversity, immuno-competence, condition factor and sexual ornamentation. Major finding of their study included: i) identification of two different allelic groups (DAB1 and DAB3) that were under different selective regimes; ii) transcription by individuals of alleles from one, or both allelic groups; iii) a high level of diversity at MHC (in 20 individuals examined 32 transcribed MHC alleles were identified); iv) no association between presence of parasite species and particular alleles; v) correlation between transcription of two DAB1 alleles with increased parasite abundance and vi) individual nucleotide diversity at either DAB1 or DAB3 increased with abundance of two parasite species.

This study aimed to characterize and measure diversity at MHC Class IIβ in the Rio Grande silvery minnow, *Hybognathus amarus*, and to examine the relationship between this variation and pathogen diversity. Three specific questions were addressed:

i) Is there an association between specific MHC alleles and prevalence and intensity of infection by pathogens?

ii) Does increased diversity at MHC confer protection from disease outbreaks?

[1] *Complementary DNA is DNA synthesized from messenger RNA in a reaction facilitated by the enzyme reverse transcriptase. cDNA sequences include exons (sequences that code for functional genes) but not introns (sequences between genes) or non-functional genes.*

iii) Is individual MHC diversity correlated with parasite species or abundance?

Methods

Sample Collection

Genetic analysis was conducted on samples collected during July fish health monitoring July 18th-July 27th 2006 and July 8th – 15th 2008. Fish were euthanized with an overdose of MS-222 (tricaine methanesulphonate). A small piece of caudal fin was removed after each fish was examined for parasites. Fin clips were preserved in 95% ethanol. Whole fish sampled for parasites were frozen and deposited at the Museum of Southwestern Biology Division of Fishes. Genomic DNA was isolated from 30 individuals per locality for genetic analysis. In addition, gill tissue was collected from the first 20 individuals examined for parasitic infections from each collection locality in 2006. In 2008 gill tissue was taken from 30 individuals. Gill tissue was preserved in RNA*later*® (Ambion) for characterization and analysis of cDNA and stored at -20°C to -80°C. Total nucleic acids, including genomic DNA and mitochondrial DNA (mtDNA) were extracted from air-dried fin clips using proteinase-K digestion and organic extraction methods (Hillis *et al.* 1996).

Microsatellites

Individuals were genotyped at seven polymorphic microsatellite loci: *Lco1*, *Lco3*, *Lco8* (Turner *et al.* 2004), *Ca6* and *Ca8* (Dimsoski *et al.* 2000), and *Ppro118* and *Ppro126* (Bessert & Orti 2003). The following pairs of loci were amplified using multiplex PCR: *Lco1/ Ca6*, *Lco3* and *Lco8* (1X PCR buffer, 2 mM MgCl2, 125µM dNTPs, 0.40-0.50 µM each primer, 0.375 units TAQ) and *Ppro118*/*Ppro126* (1X PCR buffer, 3 mM MgCl2, 125µM dNTPs, 0.40-0.50 µM each primer, 0.375 units TAQ). *Ca8* was amplified alone (1X PCR buffer, 3 mM MgCl2, 125 µM dNTPs, 0.50 µM each primer, 0.375 units TAQ polymerase). PCR cycling conditions for all loci were: one denaturation cycle of 94°C for 2 mins followed by 30 cycles of 94°C for 20s, 50°C for 20s, 72°C for 30s. For *Ppro 118*/*Ppro126* cycling conditions were one denaturation cycle of 94°C for 2 mins followed by 30 cycles of 94 °C for 20s, 60°C for 20s, 72°C for 30s. Primer concentrations in multiplex reactions were varied to facilitate equal amplification of both loci. Prior to electrophoresis 1.2µl of PCR product was mixed with 1.2µl of a solution comprised of formamide (62.5%), ABI ROX350 size standard (12.5%) and loading buffer (25%) and

Table 1. MHC Class IIβ PCR primer sequences and approximate locations.

Name	Primer Sequence (5' to 3')	Location
MHC_CYF	CAT ACT GAT GCT GTC TGC TTT	Leader peptide
FISH12S[a]	GTG GGG RRG TWT GTD GGR TAC ACT	Exon 2
EX2F	CAA ATG GAT ATT TCT ATT CTC GGT	5' Exon 2
EX2F_L4	GCT GAT GGA TAT TAT CTA GAC ATA A	5' Exon 2
Ex3R	TCA CTG AAC TGA GCT TAA CCT	5' Exon 3
LC1R	TGG TGT GGG GTA GAA GCG GT	Exon 3
LC2R	CTC TTT GGG TAG AAG TCG	Exon 3
LC4R	TCT TTC AGG GTA GAA GTC A	Exon 3
MHC2-3F	AAG GTT AAG CTC AGT TCA GT	5' exon 3
MHC2-4R	ATG GCG ATT TTA TTC CTC TC	5' exon 4

[a] Ottova et al. 2005.

denatured at 93°C for 2 minutes. Samples were run on an ABI 377 automated DNA sequencer at 50°C for 2.5 hours. *Ppro 118*/*Ppro126* and *Ca8* PCR products were run on an ABI 3100 automated capillary sequencer. One microliter of PCR product was mixed with 10µl of formamide and 0.3µl of HD400 size standard and denatured at 93°C for 5 minutes prior to loading. Genotype data was obtained using Genemapper Version 4.0 and Genescan 3.1 (Applied Biosystems).

Mitochondrial DNA- ND4

Individuals were screened for variation in a 295 base pair fragment of the mitochondrial ND4 gene using single stranded conformational polymorphism (SSCP) analysis and DNA sequencing as described in Alò and Turner (2005).

MHC- RNA Isolation and cDNA Synthesis

RNA isolations were conducted using Trizol Reagent (Invitrogen). Gill tissues were placed in 1.5 ml eppendorf tubes with 1 ml of Trizol and pulverized using a plastic pestle. Isolations were completed according to the manufacture's instructions. cDNA (complementary DNA) was synthesized from RNA using Qiagen Omniscript® or Sensiscript® Reverse Transcription kits following the manufacturer's instructions.

Primer Design

Primers that amplified MHC Class IIβ exon 3 were designed by comparing published sequences of the zebrafish and common carp (van Erp *et al.* 1996). The light strand primer (MHC2-3) was located at the beginning of exon 3 and the heavy strand primer (MHC2-4) (Table 1) was located at the beginning of exon 4. These primers amplify a fragment that encompasses exon 3, intron 3 and partial exon 4 (data not presented). Using the sequences obtained with these primers, locus specific primers were designed to amplify partial exon 3 and partial exon 2 when used in combination with previously published FishC12S (Ottova *et al.* 2005). To obtain sequences from the five prime end of Rio Grande silvery minnow exon two, a primer was designed in the leader peptide by comparing zebrafish and common carp (van Erp *et al.* 1996) sequences (*Ex2F*). Used in combination with *MHC2-4*, sequences were obtained for entire exon 2 allowing the design of primers that amplified this exon. A 3' primer was also designed based on Rio Grande silvery minnow sequences that would amplify exon 2 regardless of which allelic group was present in the individual.

Allele screening

Individual cDNA samples were screened for the presence of each allelic group using group specific primer combinations. DAB1 alleles were amplified (1X Genescript Taq polymerase buffer, 3mM $MgCl_2$, 125µM dNTPs, 0.35µM each primer [*MHC Ex2F* and *Lc1R*], 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 for 20s, 72°C for 20s and a final extension step of 72°C for 10 minutes. DAB1(2) alleles (see results) were amplified (1X Genescript Taq polymerase buffer, 3mM $MgCl_2$, 125µM dNTPs, 0.35µM each primer [*MHC Ex2F* and *Lc2R*], 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, 56°C for 20s, 72°C for 20s and a final extension step of 72°C for 10 minutes. DAB3 alleles were amplified (1X Genescript Taq polymerase buffer, 3mM $MgCl_2$, 125µM dNTPs, 0.35µM each primer [M*HC Lc4F* and *Lc4R*] 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, 58°C for 20s, 72°C for 20s and a final extension step of 72°C for 10 minutes. For individuals that failed to amplify using the primer combination given above, *MHC Ex2F* or *MHC Lc4F* was used in conjunction with MHC-Ex3R (1X Genescript Taq polymerase buffer, 3mM $MgCl_2$, 125µM dNTPs, 0.35µM each primer, 0.375 units Taq polymerase). Screening reactions were conducted in 10µL volumes.

Single Stranded Conformational Analysis

Samples that were positive for each of the allelic groups were then 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% bromphenol blue, 0.05% xylene cyanol FF) was added to the remaining 10µL of PCR product. Samples were denatured at 93°C for 5 minutes and immediately quenched on ice. Fifteen microliters of each sample were loaded on a non-denaturing polyacrylamide minigel (5% acrylamide for allelic groups DAB1(2) and DAB3 and 6% for DAB1) (with 50% glycerol). Samples of known haplotype were included to assist in haplotype 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 DAB1(2) and 4.5 hours for groups DAB1 and DAB3. Gels were stained in ethidium bromide and visualized on a UV transilluminator.

Gel plugs were removed from the gel using a razorblade from each variant on the gel. Gel plugs were resuspended in 30μl of deionized water and 30μl of TE and incubated at 37°C shaking for 2-3 hours. Samples were then centrifuged for 20 minutes at 30,000 RPM and the supernatant removed and placed in a new 1.7 ml centrifuge tube. 500 μL of 100% ethanol was added and the tube then placed at -80°C for 15 minutes to precipitate the DNA. Samples were centrifuged for 20 mins at 30,000 RPM to pellet the DNA. The pellet was dried at room temperature and then resuspended in 50μL of TLE. One microliter of this was used in PCR reactions 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). Sequences were visualized and checked using Sequencher Version 4.6 (Gene Codes Corp).

Cloning

For individuals that appeared to be heterozygotes (and for a portion of homozygotes) PCR products were cloned using the Promega pGEM-T Easy Vector kit following the manufacturer's directions. Plasmid DNA was purified using a boil preparation method (Sambrook *et al.* 1989). Between four and eight clones were sequenced per individual. MHC sequences were aligned manually using Se-Al Version 2.0a11 (available at http://evolve.zoo.ox.ac.uk/software.html?id=seal). Sequences were designated as different alleles if they were encountered more than once and in multiple clones. Sequences differing by one or two base pairs from previously designated haplotypes were not designated as new alleles unless they were encountered multiple times, as they were presumed to be an artifact of PCR. Nomenclature of alleles followed Klein *et al.* (1990). The reason for this conservative approach to allele designations is that MHC sequences are prone to formation of artifactual, mosaic sequences during the cloning procedure (Borriello & Krauter 1990; L' Abbe *et al*. 1992).

Statistical Analysis- Microsatellites

Microsatellite data was checked for errors using MICROSATELLITE TOOLKIT (add-in for Microsoft Excel written by S. Park, available at http://animalgenomics.ucd.ie/sdepark/ms-toolkit/). Nei's unbiased genetic diversity (Nei

1987), observed heterozygosity and allele frequencies were obtained using this program. The computer program Microchecker (van Oosterhout *et al.* 2004) was used to examine the data for scoring errors due to stuttering, presence of large allele dropout and null alleles. For each microsatellite locus and population, allelic richness (A_R), total number of alleles (N_a) and inbreeding co-efficients (F_{IS}) were obtained using FSTAT version 2.9.3.1 (Goudet 1995). Allelic richness was calculated using the methods described Petit *et al.* (1998). This method allows the number of alleles to be compared among populations independently of sample size (Leberg 2002) and is based on the smallest number of individuals typed for any locus. The computer package ARLEQUIN (Schneider *et al.* 2000) was used to assess whether there were significant departures from Hardy-Weinberg equilibrium using the procedure of Guo and Thompson (1992). Global tests for linkage disequilibrium (non-random association of loci) were conducted for all pairs of loci using FSTAT. Bonferroni (Rice 1989) correction was applied to account for multiple simultaneous tests. Weir and Cockerham's (1984) *F*-statistics were calculated using ARLEQUIN (Schneider *et al.* 2000) to determine the magnitude of differences between samples collected at different localities. F_{ST} is the standardized variance in allele frequencies between populations and is the most commonly used measure of genetic distance between populations. P-values for all statistics were generated using a bootstrapping method (10,000 permutations).

Mt-DNA- ND4

Estimates of unbiased gene diversity (h) and nucleotide diversity (π) were obtained using ARLEQUIN Version 3.0 for mitochondrial DNA data. Allelic richness was calculated using FSTAT. ϕ-statistics were calculated from mt-DNA data using ARLEQUIN (Excoffier *et al.* 1992). ϕ-statistics are equivalent to F-statistics (Weir & Cockerham 1984), however they incorporate allele frequencies and evolutionary distances between haplotypes.

Phylogenetic Analysis- MHC

MHC sequences were aligned manually using Se-Al Version 2.0a11 (available at http://evolve.zoo.ox.ac.uk/software.html?id=seal). The computer program TCS (Clement *et al.* 2000) was used to construct a statistical parsimony network among MHC alleles using the method of Templeton *et al.* (1992). We aligned Rio Grande silvery minnow MHC sequences to published MHC sequences from representatives of the cyprinids

including common carp, Lake Tama African 'large' barb, carp bream and zebrafish. Phylogenetic trees were reconstructed using Bayesian analysis as implemented in MrBayes (Huelsebeck and Ronquist 2001). For Bayesian analysis the General Time Reversible Model + I + Γ was used and model parameter were treated as unknown variables with uniform priors and were estimated during the analysis. Random starting trees were used and two simultaneous analyses were run for 1 x 10^7 generations sampling every 100^{th} generation from the chain. Four chains (three hot and one cold) were run simultaneously. The first 25% of trees were discarded as burn-in samples and the remaining trees were used to construct a majority rule consensus tree. Bayesian posterior probabilities were obtained for the remaining tree. Posterior probabilities above 95% are considered strong support for a clade (Wilcox *et al.* 2002).

MHC- *Tests of Selection*

To determine whether there was evidence of selection and the type of selection (positive [beneficial mutations increase in frequency in the population], purifying [deleterious alleles decrease in frequency in the population], or neutral [i.e., no evidence of selection]) sequences were examined at the amino acid level. If MHC sequences are evolving neutrally, the ratio of non-synonymous (causes an amino acid substitution) (d_N) to synonymous (does not result in an amino acid substitution) (d_S) will not differ significantly from one. If there is positive selection, d_N with be greater than d_S, whilst for purifying selection, d_N will be less than d_S. We conducted Z-test of selection as implemented in MEGA v. 3.1. Ratios of d_S to d_N were estimated for sites presumed to be involved in peptide binding and for non-peptide binding sites. Designation of peptide binding sites were based on comparisons to human HLA three dimensional structure (Brown *et al.* 1993) following Dixon *et al.* (1996) and Ottova *et al.* (2005; 2007) because peptide binding sites have not been determined specifically for fish, including cyprinid fishes. Ratios of d_S to d_N were estimated across all MHC alleles identified and within each of the major allelic groups.

Association between MHC and parasite diversity

Scatter plots were generated to examine the relationship between sampling locality and genetic and parasite diversity measures including allelic richness (Mt-DNA and microsatellites), total number of MHC alleles, average number of parasite species and average level of infection of individuals. Non-parametric Spearman's rank correlation was used to test for associations

between MHC diversity (measured by total number of alleles observed irrespective of loci), total number of allelic groups represented (an allelic group was defined as a distinct lineage as determined by phylogenetic analysis) and parasites (measured by number of species observed per individual) using the computer package SPSS. For comparisons between the genetic data and parasites only gill parasite data was used. Allelic groups were DAB1, DAB1(2) and DAB3. The designation of these groups was based on the results of phylogenetic analysis. Intensity of infection was obtained per fish by categorizing abundance estimates: 0 (uninfected), 1 (less than 10 individuals of a parasite species), 2 (less than 50 individuals of a parasite species), 3 (more than 50 individuals of a parasite species). To obtain an overall estimate of the level of infection across all parasite species per individual, category values were summed (hence level of infection could range from 0 to 15 given a total of five parasite species).

Results

Microsatellite Diversity

All measures of genetic diversity calculated from microsatellite DNA data were similar between samples collected at each locality in 2006 (the only exception was WJR06-464 where fewer individuals were collected). In 2006 allelic richness was the greatest in the sample WJR06-462 (A_R = 9.363) while observed heterozygosity was highest in the sample SRD06-45 (H_O = 0.738) (Table 2). In 2008, all measures of genetic diversity were similar between localities. Allelic richness was slightly lower in the sample WJR08-814 (A_R = 9.586). In 2008 observed heterozygosity ranged from 0.659 to 0.720 (Table 2). In 2006 there were 10 departures from Hardy-Weinberg equilibrium whilst there were 13 deviations in 2008 from 42 comparisons. All of these were explained by deficiencies of heterozygotes. There was no evidence of linkage disequilibrium between pairs of microsatellite loci.

Pairwise F_{ST} was calculated between samples collected at six different localities on the Rio Grande (Table 3a). In 2006, F_{ST} values were very small and not significantly different from zero (at nominal α = 0.05). In 2008, there were six significant comparisons from 21 comparisons after Bonferroni correction was applied.

MtDNA Diversity-

In 2006 sequence data for partial mitochondrial DNA ND4 gene (295 base pairs) was obtained for 163 individuals allowing eight haplotypes to be identified. In 2008 sequence

Table 2. Summary statistics by collection locality for 2006 and 2008. Sample size (*N*), number of alleles (N_a), allelic richness (A_R) expected heterozygosity (H_E), observed heterozygosity (H_O), and average weighted inbreeding co-efficient (F_{IS}), gene diversity (*h*) and nucleotide diversity (π) are given. For MHC sample size (*N*), number of alleles (N_a) are given for DAB1-DAB1(2) and for DAB3.

		ND4					DAB1		DAB3	Microsatellites					
Field Number	**Locality**	**N**	**Na**	**A_R**	***h***	***π***	**N**	**Na**	**Na**	**N**	**Na**	**A_R**	***H_O***	***H_e***	***F_{IS}***
WJR06-468	**Bernalillo**	30	6	5.397	0.646	0.004	21	22	2	30	14.29	9.214	0.722	0.86	0.164
WJR08-818		30	4	3.459	0.313	0.003	26	17	5	30	14	10.36	0.72	0.849	0.155
WJR06-466	**Alameda**	30	6	5.547	0.687	0.006	19	19	2	30	14.57	8.824	0.698	0.846	0.178
WJR08-817		29	5	4.046	0.466	0.003	25	21	2	30	14.78	10.72	0.684	0.867	0.214
WJR06-464	**Los Padillas**	13	3	3.000	0.295	0.002	13	11	2	13	9.57	8.22	0.739	0.847	0.133
WJR08-816		14	4	4.000	0.703	0.005	12	18	3	17	11.11	10.21	0.659	0.864	0.243
WJR06-462	**Los Lunas**	30	5	4.042	0.041	0.003	20	19	2	30	14.71	9.363	0.708	0.884	0.205
WJR08-815		29	6	5.146	0.584	0.002	25	20	3	30	13.67	10.23	0.697	0.844	0.177
WJR06-461	**La Joya**	30	5	4.466	0.457	0.003	18	10	3	30	15.57	9.35	0.742	0.857	0.136
WJR08-814		29	5	4.344	0.372	0.003	28	23	4	30	13	9.586	0.682	0.842	0.194
SRD06-045	**San Antonio**	30	7	6.276	0.632	0.004	20	17	0	30	14	8.796	0.738	0.863	0.147
WJR08-813		29	4	3.474	0.446	0.003	25	16	4	30	13.67	10.06	0.712	0.84	0.155

Table 3. Pairwise values of F_{ST} (below diagonal) and associated P-values (above diagonal) calculated for microsatellite DNA data (a) for 2006 and for (b) 2008 and ϕ_{ST} for mtDNA-ND4 (c) for 2006 and for (d) 2008.

(a)

usats-F_{ST}	SRD06-045	WJR06-461	WJR06-462	WJR06-464	WJR06-466	WJR06-468
SRD06-045	-	0.6611	0.5303	0.4697	0.9854	0.6074
WJR06-461	-0.0030	-	0.5703	0.8975	0.6406	0.4189
WJR06-462	-0.0011	-0.0018	-	0.7940	0.1846	0.1006
WJR06-464	0.0006	-0.0098	-0.0082	-	0.1836	0.2920
WJR06-466	-0.0092	-0.0025	0.0047	0.0072	-	0.5576
WJR06-468	-0.0014	0.0012	0.0079	0.0058	-0.0011	-

(b)

usats-ϕ_{ST}	WJR08-818	WJR08-817	WJR08-816	WJR08-815	WJR08-814	WJR08-813
WJR08-818	*	0.00195	0.00488	0	0.0498	0
WJR08-817	0.01586	*	0.8877	0.00977	0.00293	0.09668
WJR08-816	-0.00007	0.01997	*	0.0332	0.66895	0.05566
WJR08-815	0.01235	0.00155	0.01418	*	0	0.79492
WJR08-814	0.00756	0.01431	0.01153	-0.00053	*	0.00293
WJR08-813	0.02509	0.00948	0.03562	0.01919	0.01537	*

(c)

ND4-F_{ST}	SRD06-045	WJR06-461	WJR06-462	WJR06-464	WJR06-466	WJR06-468
SRD06-045	-	0.4844	0.2588	0.1953	0.3955	0.9902
WJR06-461	-0.0094	-	0.9990	0.6016	0.0752	0.4346
WJR06-462	0.0071	-0.0290	-	0.6172	0.0264	0.2754
WJR06-464	0.0156	-0.0189	-0.0190	-	0.0850	0.1962
WJR06-466	-0.0044	0.0471	0.0788	0.0746	-	0.3350
WJR06-468	-0.0286	-0.0070	0.0048	0.0255	0.0022	-

(d)

ND4-ϕ_{ST}	WJR08-818	WJR08-817	WJR08-816	WJR08-815	WJR08-814	WJR08-813
WJR08-818	0	0.26562	0.39355	0.18945	0.75879	0.16602
WJR08-817	0.00216	0	0.11426	0.28125	0.7041	0.89062
WJR08-816	-0.00438	0.05241	0	0.1748	0.19336	0.03418
WJR08-815	0.01662	0.01317	0.02538	0	0.39453	0.08594
WJR08-814	-0.02424	-0.0177	0.01836	0.00158	0	0.45508
WJR08-813	0.0329	-0.02454	0.09087	0.04396	-0.0024	0

data was obtained for 158 individuals revealing eight haplotypes. In all populations, haplotype A was the most prevalent. In 2006, three to seven haplotypes were identified per locality and in 2008 four to six haplotypes were seen at each locality.

In both 2006 and 2008 one significant value of Φ_{ST} was identified between WJR06-462 and WJR06-466 (F_{ST} = 0.079, P = 0.0264) and between WJR08-813 and WJR08-816 (F_{ST} = 0.0908, P = 0.0371) (Table 3b). After Bonferroni correction (Rice 1989) for multiple comparisons neither of these remained significant.

MHC Diversity- Overview

Two-hundred and seventy-four base pairs of sequence data encompassing 91 codons of the exon two region of MHC Class II beta region (the full length of exon 2 is 95 codons). Conserved cysteine residues (which are involved in the formation of di-sulfide bridges) and N-linked glycosylation sites were identified based on comparison to other published cyprinid sequences. These conserved sites could be identified in all of the MHC alleles observed in Rio Grande silvery minnow. Seventy-two unique haplotypes were identified among the 252 individuals. Most haplotypes were rare with only ten and eleven haplotypes present in more than 5% of individuals in 2008 and 2006 respectively (Figures 1a and 1b). In 2008, allele Hyam-DAB3*06 was present in more than 10% of individuals; an increase in the frequency in 2006. Eighteen alleles were identified that had a single codon deletion at position 72 (numbering based on Ottova *et al.* 2005). These alleles all belonged to the DAB1(2) group (see section below on phylogenetic analysis). A single haplotype belonging to the DAB1 group had a deletion at this position.

Phylogenetic Analysis

Bayesian analyses revealed two highly divergent clusters of MHC alleles. One of these was similar to DAB1 group of alleles identified previously in *Danio rerio*, *Cyprinus carpio*, *Barbus intermedius* (Ono *et al.* 1993). The other group was more similar to the DAB3 alleles (van Erp *et al.* 1993) previously described in other members of the family Cyprinidae than to DAB1 alleles identified in Rio Grande silvery minnow (Figure 2). The DAB3 group of alleles identified in Rio Grande silvery minnow formed monophyletic groups with *D. rerio*, *C. carpio*, *B. intermedius* DAB3-DAB4 alleles (100% posterior probability). Relationships among the Hyam-DAB3 alleles were poorly resolved. In addition to the DAB3 group of alleles, two other allelic groups were identified in Rio Grande silvery minnow that each had high to moderate support. The most basal

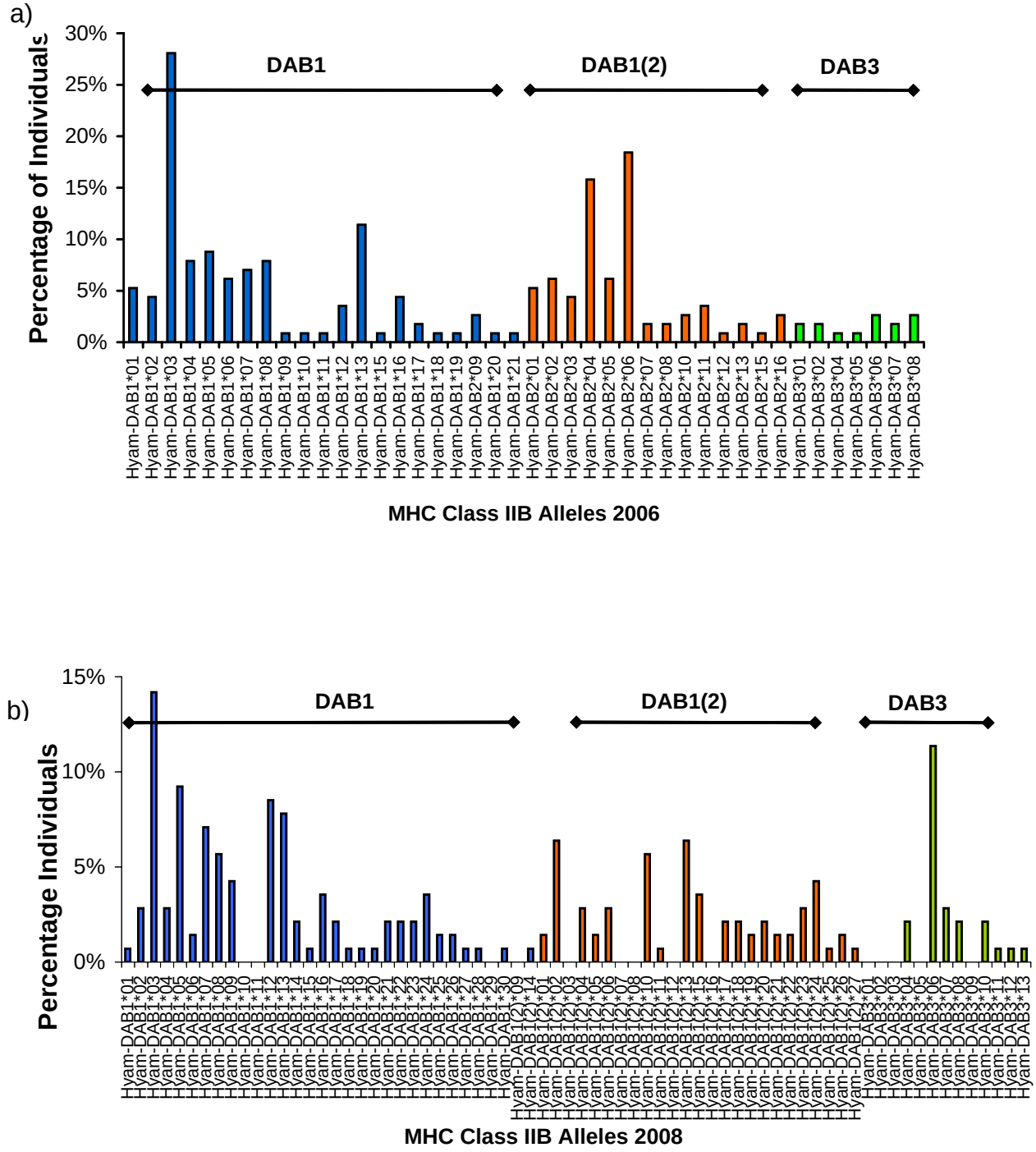


Figure 1. Percentage of individuals identified with each MHC Class IIB allele. Allelic groups (DAB1, DAB1(2) and DAB3) to which alleles belong are given above.

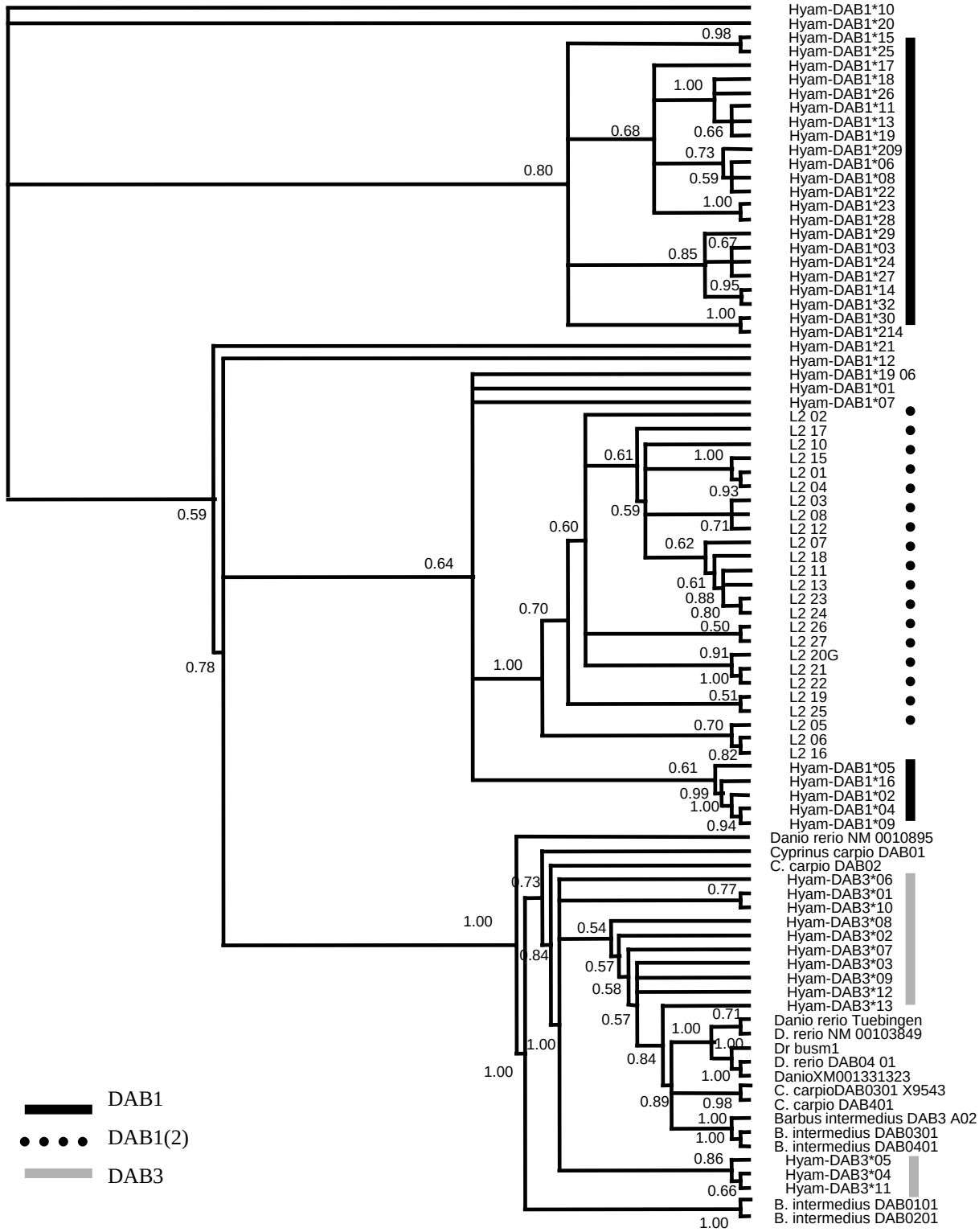
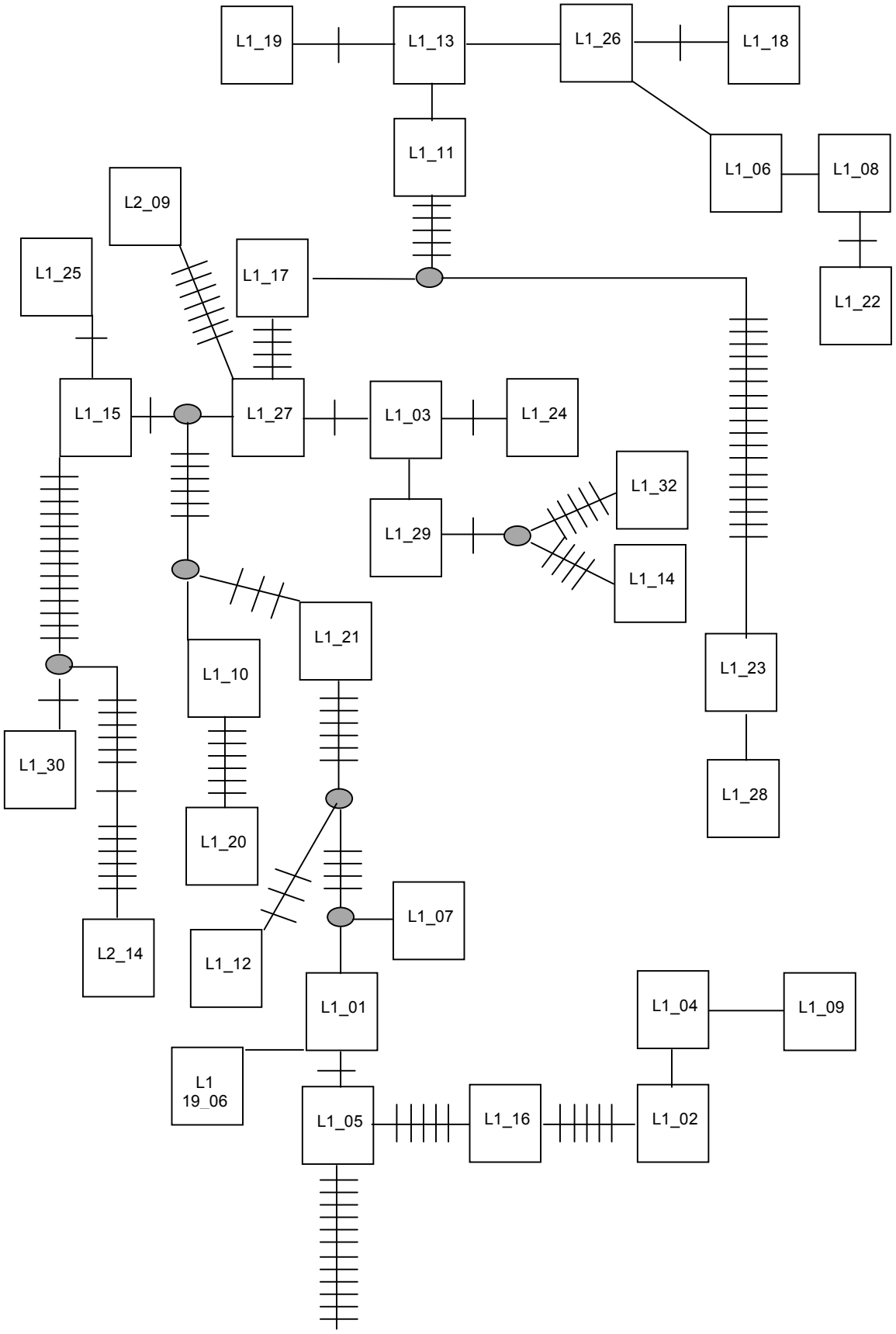


Figure 2. Bayesian phylogenetic tree of MHC Exon 2 Rio Grande silvery minnow alleles. Values by the branches are posterior probabilities.


L1_19
L1_13
L1_26
L1_18
L1_11
L1_06
L1_08
L2_09
L1_25
L1_17
L1_22
L1_15
L1_27
L1_03
L1_24
L1_32
L1_29
L1_14
L1_21
L1_10
L1_23
L1_30
L1_28
L1_20
L1_07
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L1
19_06
L1_05
L1_16
L1_02

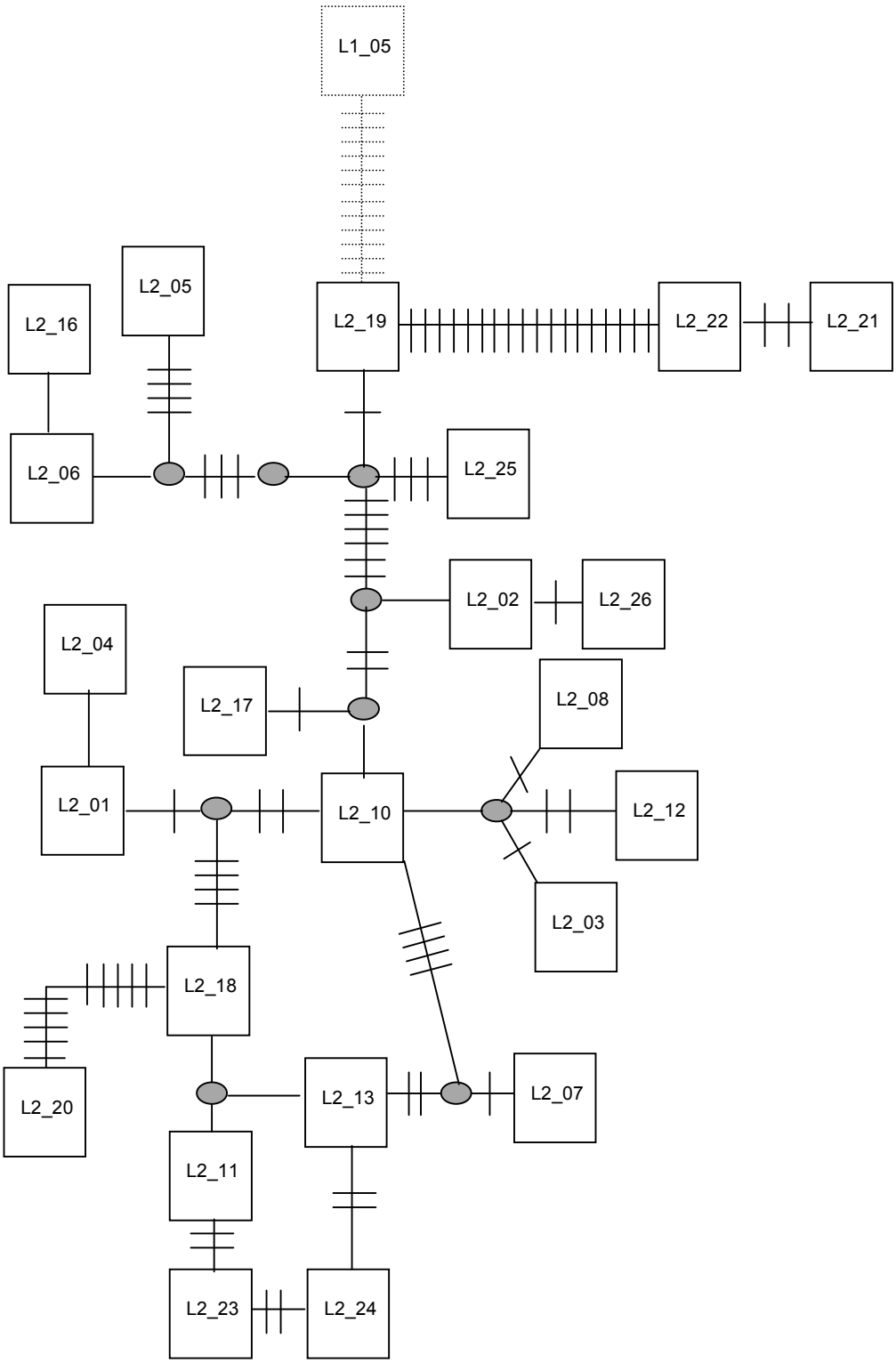


Figure 3a. Statistical phylogenetic network of DAB1 and DAB1(2) haplotypes identified in Rio Grande silvery minnow. Ovals and bars represent single nucleotide changes among haplotypes (indicated by boxes). Haplotype Hyam-DAB1*5 is shown on both pages and links the networks.

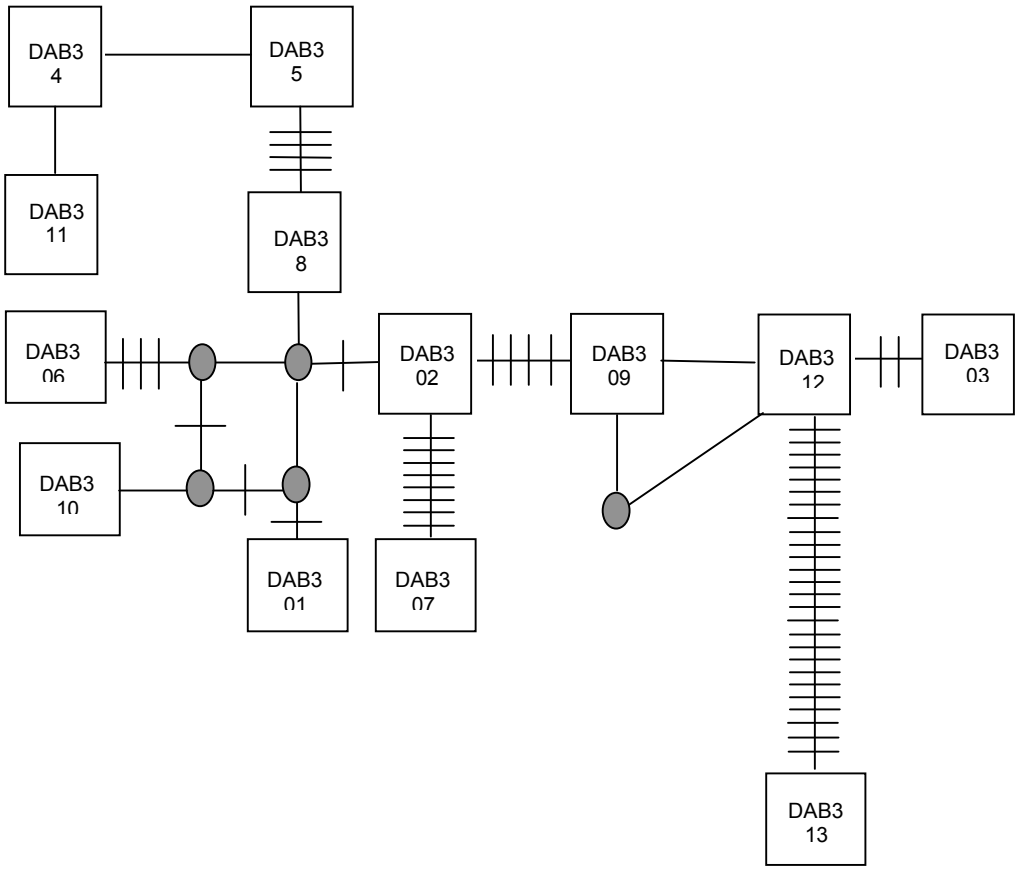


Figure 3b. Statistical phylogenetic network of DAB3 alleles identified in Rio Grande silvery minnow. Ovals and bars represent single nucleotide changes among haplotypes (indicated by boxes).

of the silvery minnow DAB1 groups included 22 alleles (80% posterior probability). The next group contained 55 alleles (61% posterior probability). The position of 10 DAB1 alleles was less well resolved with respect to these well-supported groups. The last of these groups was DAB1(2) (100% support), containing 25 different alleles which had 100% posterior probability support. The relationships identified by the statistical phylogenetic networks were consistent with the Bayesian results. Statistical phylogenetic networks demonstrate the high number of nucleotide substitutions among haplotypes within both the DAB1 and DAB1(2) groups (Figure 3a-b). In 2006, eight haplotypes were identified within the DAB3 group from 274 base pairs of sequence. In 2008, seven haplotypes were identified belonging to this group. Haplotype Hyam-DAB3*13 was very distinct from the other DAB3 alleles. Within this group DAB03*06 was the most frequently encountered allele in 2006 and in 2008. Divergence among the DAB3 alleles is much less than either of the DAB1 groups.

Evidence of Selection

When all sites and all alleles were considered, neutrality was rejected in favor of positive selection (Table 4). This was the case when only peptide binding sites and non-peptide binding sites were considered, and when all DAB1 alleles were considered. Interestingly, neutrality could not be rejected for DAB3 sequences when only peptide binding sites were considered but there was evidence of positive selection when all sites were considered and for non-peptide binding sites. For consideration of DAB1(2) alleles only, positive selection was evident when all sites were included but not when data was partitioned into sites involved with peptide binding or non-peptide binding.

MHC Diversity- Individuals and Allelic Groups

Individuals expressed alleles that belonged to one, two or three different allelic groups. In 2006, the maximum number of alleles identified per individual was five and in 2008 was it was four alleles. The mean number of alleles expressed per individual was 1.9 (Std. Dev. = 0.93) in 2006 and in 2008, the mean was 1.4 (Std. Dev.= 0.90) (Figure 4). Based on the phylogenetic analysis of alleles, individuals could be placed into the following groups: 1) individuals that expressed one or multiple alleles from a single allelic group [DAB1, DAB1(2) or DAB3]; 2) individuals that expressed one or multiple alleles from multiple allelic groups [DAB1 & DAB1(2); DAB1 & DAB3; DAB1(2) & DAB3] and 3) individuals that did not express alleles (or expression was not detected) in any allelic group. In 2006, four individuals were identified that did not appear to express (or expression was not detected) any MHC allele. RNA may have been degraded in

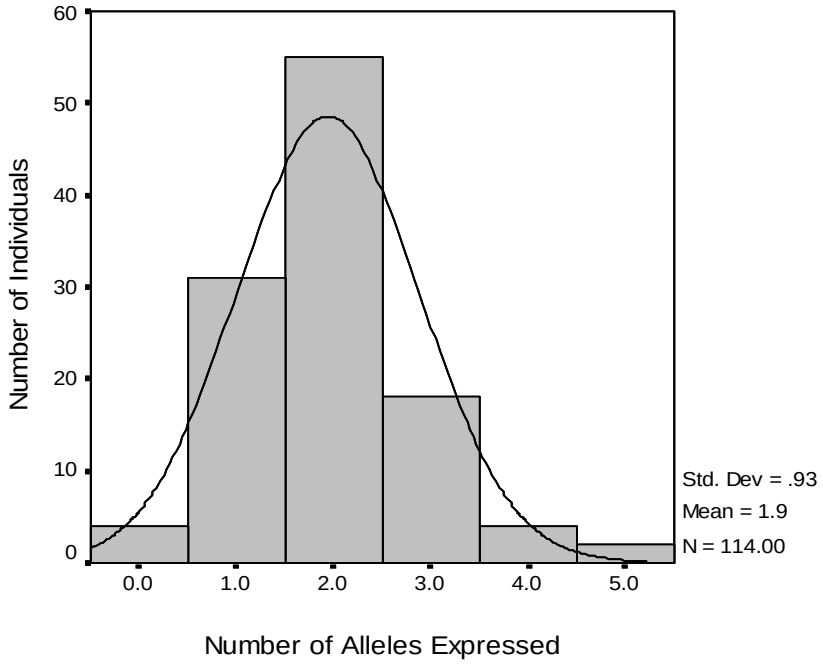
these samples. Using genomic DNA as template however, a PCR product was obtained for each of these individuals. In 2008, we could not determine MHC alleles for 23 individuals. Amplification of MHC was either unsuccessful due to absence of MHC expression or due to degradation of RNA or in some cases amplification was successful but sequence was not obtained. In 2006 and 2008 around 5% of fish expressed alleles representing each of the three allelic lineages. In 2006 the majority of fish (57%) expressed alleles belonging to two different groups, whilst 34% expressed alleles belonging to a single group. In 2008, the majority of individuals expressed alleles belonging to two allelic groups (59%) whilst 43.1% expressed alleles from a single group. In 2006 and 2008, 96% of individuals expressed alleles belonging to the DAB1- DAB1(2) group. In 2006 alleles belonging to the DAB3 group were only detected in 10.5% of individuals and only a single allele per individual (i.e. all individuals were homozygous) was identified within this allelic group. In 2008, we identified alleles from this group in 23% of individuals.

The average number of amino acid substitutions within each of the main allelic groups was 14.191 (SE = 1.936) substitutions for DAB1 alleles, 9.383 (SE = 1.558) among DAB1(2) alleles and 9.244 (SE = 1.757) among DAB3 alleles. There was an average of 20.520 (SE = 2.853) amino acid substitutions between the DAB1(2) group of alleles and the DAB1 group, whilst between these groups and the DAB3 group there were 46.244 (SE = 4.226) and 46.597 (SE = 4.310) amino acid changes, respectively. The number of amino acid changes between all Rio Grande silvery minnow the three DAB groups of alleles and outgroup alleles was 42.218 (DAB1), 42.391 [DAB1(2)] and 34.308 (DAB3).

Association between MHC and parasite diversity

In 2006, no correlation was found between the number of MHC alleles or the number of allelic groups and parasite diversity or intensity of infection (Table 5), nor was there an association between MHC and bacterial diversity. Spearman's Rho was significantly positive (after Bonferroni correction) for comparisons between populations (sampling locality) and parasite diversity (rho = 0.341, $P < 0.0001$), the number of MHC alleles identified per fish and the number of allelic groups (rho = 0.195, $P < 0.0001$) and between the parasite diversity and infection intensity. In 2008, Spearman's Rho was significantly negative for comparisons between collection locality and parasite diversity and infection intensity and for the comparison between parasite diversity and bacterial diversity. Spearman's Rho was significantly positive for the comparison of collection locality and bacterial diversity.

a)



b)

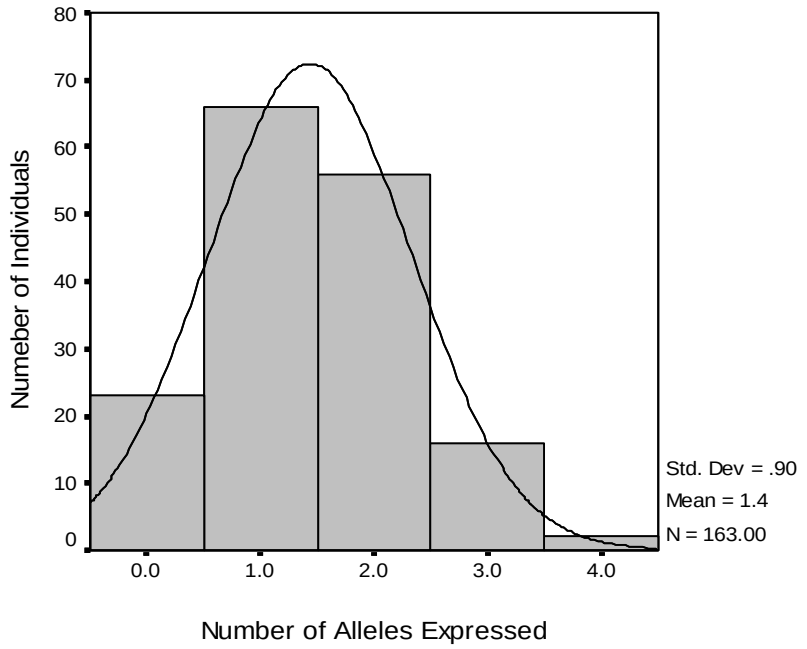


Figure 4. Number of alleles expressed per fish in a) 2006 and b) 2008.

Table 4. The number of sequences (N), number of non-synonymous changes per non-synonymous site (d_N), number of synonymous changes per synonymous site (d_S) and standard errors (SE) associated with these for all alleles, and by allelic groups [DAB1, DAB1(2) and DAB3] and for all sites, peptide binding sites. The ratio of d_N to d_S and results of Z-tests (where the null hypothesis is $d_N=d_S$) (and associated P-values) are given. Significant values are shaded.

		All Alleles	**SE**	**DAB1 & DAB1(2)**	**SE**	**DAB01**	**SE**	**DAB1(2)**	**SE**	**DAB03**	**SE**
	N	73		60							
All-Sites	dN	0.2412	0.0316	0.1282	0.0218	0.1000	0.0169	0.0677	0.0133	0.0613	0.0118
	dS	0.1036	0.0195	0.0342	0.0111	0.0229	0.0101	0.0258	0.0091	0.0214	0.0101
	dN/dS	2.3282		3.7485		4.3668		2.6240		2.8645	
	dN≠dS	**4.3821**	P=0.0000	4.9102	P=0.0000	4.4016	P=0.0000	2.8427	P=0.0053	2.4919	P=0.0141
PBR Only	dN	0.5025	0.0936	0.3024	0.0735	0.2603	0.0572	0.1468	0.0430	0.0802	0.0270
	dS	0.2381	0.0800	0.0872	0.0361	0.0813	0.0349	0.0352	0.0211	0.0469	0.034
	dN/dS	2.1105		3.4679		3.2017		4.1705		1.7100	
	dN≠dS	**2.7185**	P=0.0075	3.4395	P=0.0008	3.0874	P=0.0025	2.6577	P=0.0089	0.7813	P=0.435
Non-PBR	dN	0.1787	0.0296	0.0843	0.0187	0.0660	0.0149	0.0435	0.0113	0.0551	0.0131
	dS	0.0681	0.0189	0.0225	0.0095	0.0151	0.0094	0.0228	0.0100	0.0125	P=0.0083
	dN/dS	2.6241		3.7467		4.3709		1.9079		4.4080	
	dN≠dS	3.0989	P=0.0024	3.2781	P=0.0014	2.7375	P=0.0071	1.5340	P=0.1276	2.6170	P=0.0100

Table 5. Spearman's rho is given below the diagonal and associated P-Values are given above the diagonal for a) 2006 and b) 2008. Significant values are shaded (P < 0.05).

a)

rho	Collection Locality	# Parasite Species	# Bacteria Species	Infection Intensity	# Allelic Groups	Number MHC Alleles
Collection Locality	*	0.0002	0.4227	0.0301	0.7746	0.0375
# Parasite Species	0.3414	*	0.5278	0.0000	0.0787	0.1471
# Bacteria Species	0.0758	-0.0597	*	0.0982	0.3673	0.0834
Infection Intensity	0.2032	0.8465	-0.1557	*	0.1572	0.1464
# Allelic Groups	0.0271	-0.1654	0.0852	-0.1334	*	0.0000
# MHC Alleles	0.1951	-0.1366	0.1629	-0.1369	0.7972	*

b)

rho	Collection Locality	# Parasite Species	# Bacteria Species	Infection Intensity	# Allelic Groups	Number MHC Alleles
Collection Locality	*	0.000	0.000	0.000	0.930	0.685
# Parasite Species	-0.634	*	0.000	0.000	0.655	0.958
# Bacteria Species	0.394	-0.329	*	0.000	0.691	0.549
Infection Intensity	-0.608	0.929	-0.293	*	0.795	0.962
# Allelic Groups	0.007	0.035	-0.031	0.020	*	0.000
# MHC Alleles	0.032	0.004	-0.047	-.004	0.900	*

Error bar plots of the mean level of infection in fish with the presence or absence of the five most common alleles were constructed (2006 and 2008 data were pooled). These suggested that there may be significant associations between particular alleles and parasites (Figure 5a-e). The expression of Hyam-DAB1*05 was associated with lighter *Costia* spp. Infection, whilst expression of Hyam-DAB2*06 was associated with heavier *Costia* spp. infection. Expression of Hyam-DAB1*13 was associated with heavier *Apiosoma* infection. No relationship was detected between presence or absence of Hyam-DAB1*03 or Hyam-DAB2*04 and infection by a particular species.

Relationship of sampling locality, parasites and genetic diversity- 2006

There was no noticeable difference among sampling localities in the amount of neutral genetic diversity (measured at the mtDNA-ND4 locus and across microsatellite loci) (Figure 6a). In contrast, there were more alleles expressed at MHC Class IIβ in fish collected from Bernalillo (WRJ06-468) and Alameda (WRJ06-466) when compared to fish collected from the southern sampling localities (La Joya and San Antonio). There were no strong trends in the number of parasite species or the level of infection between sampling localities. Nevertheless, overall level of infection appears to be higher in fish collected from the northern sites, whilst more parasite species were found on fish collected from Los Lunas (WJR06-462) and La Joya (WJR06-461). Fish collected from Los Padillas had the highest average level of infection.

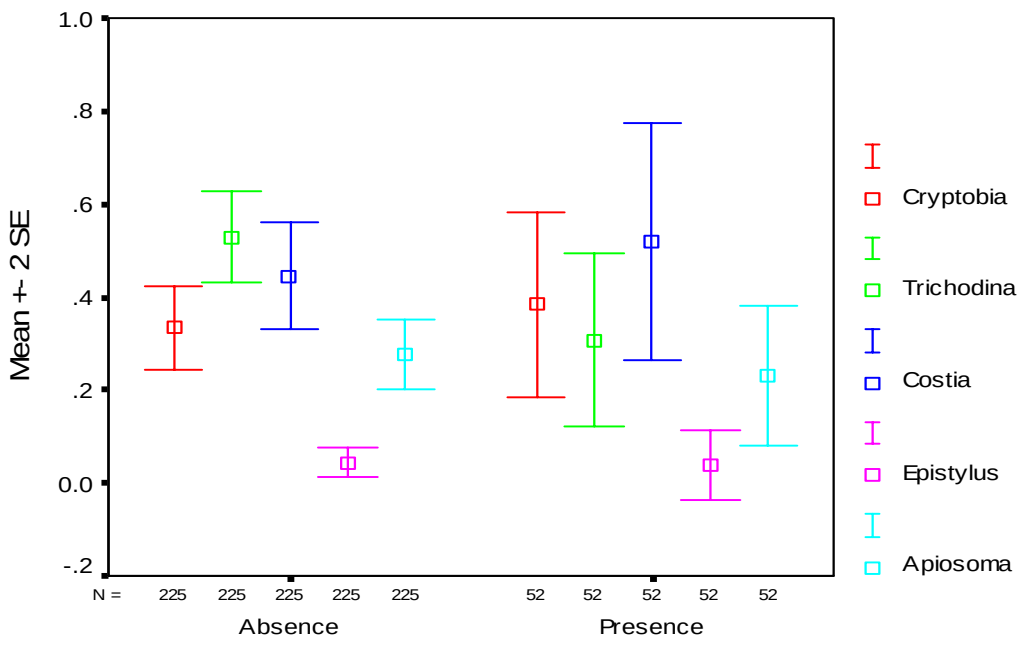
Relationship of sampling locality, parasites and genetic diversity- 2008

In 2008, there was no difference in the amount of neutral genetic diversity as measured by allelic richness and total number of ND4 alleles among collection localities (Figure 6b). There were also no clear trends in the number of MHC alleles detected and collection locality. There was a striking trend in the average number of parasite species found on individuals by collection locality, with few species and very low infection levels at the two most northern sites (Bernallilo and Alameda), moderate infection levels and nubmber of species at Los Padillas and higher numbers of species and levels of infection on fish from Los Lunas, La Joya and Los Padillas.

Discussion

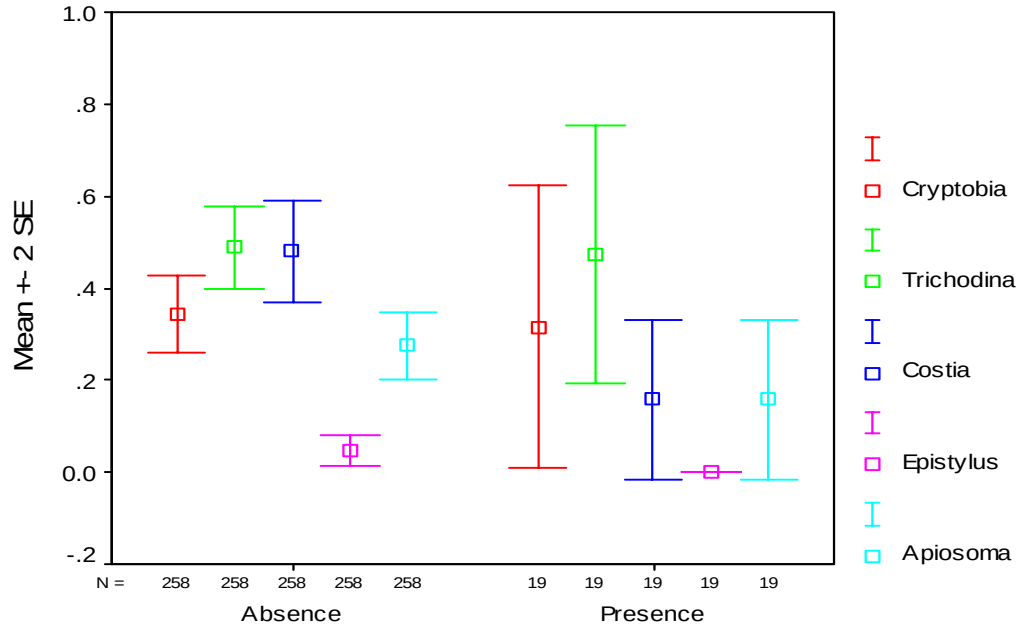
The goals of this study were to describe genetic variation in the endangered Rio Grande silvery minnow at loci that form a crucial component of the adaptive immune system and

a)



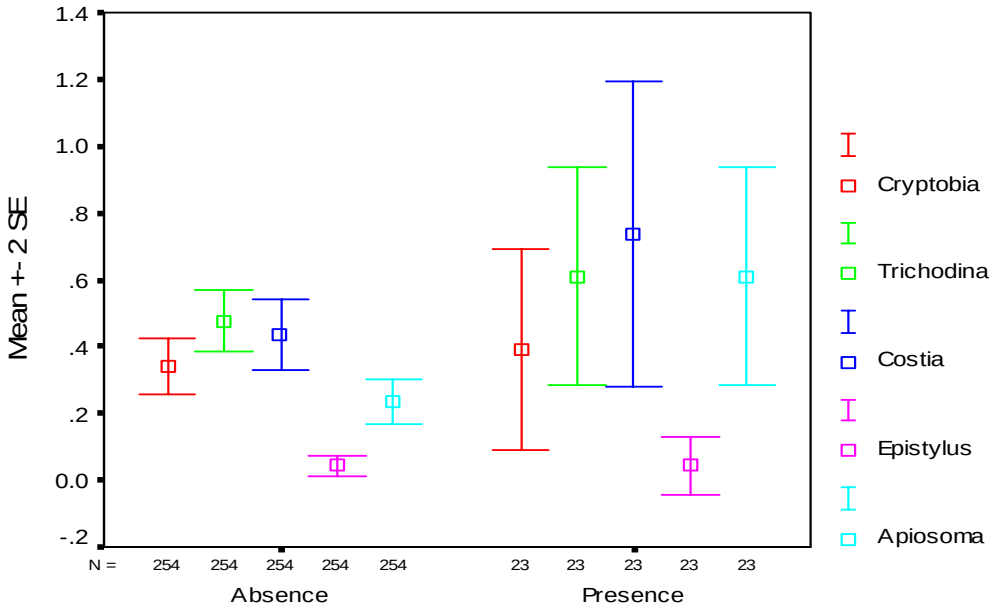
DAB1_3

b)



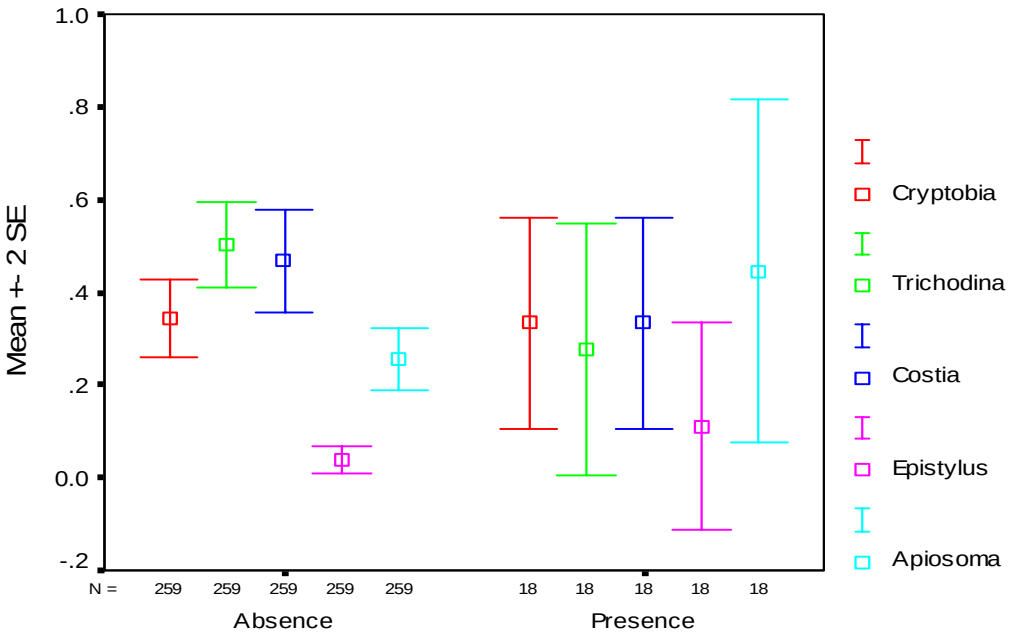
DAB1_5

c)



DAB1_13

d)



DAB2_4

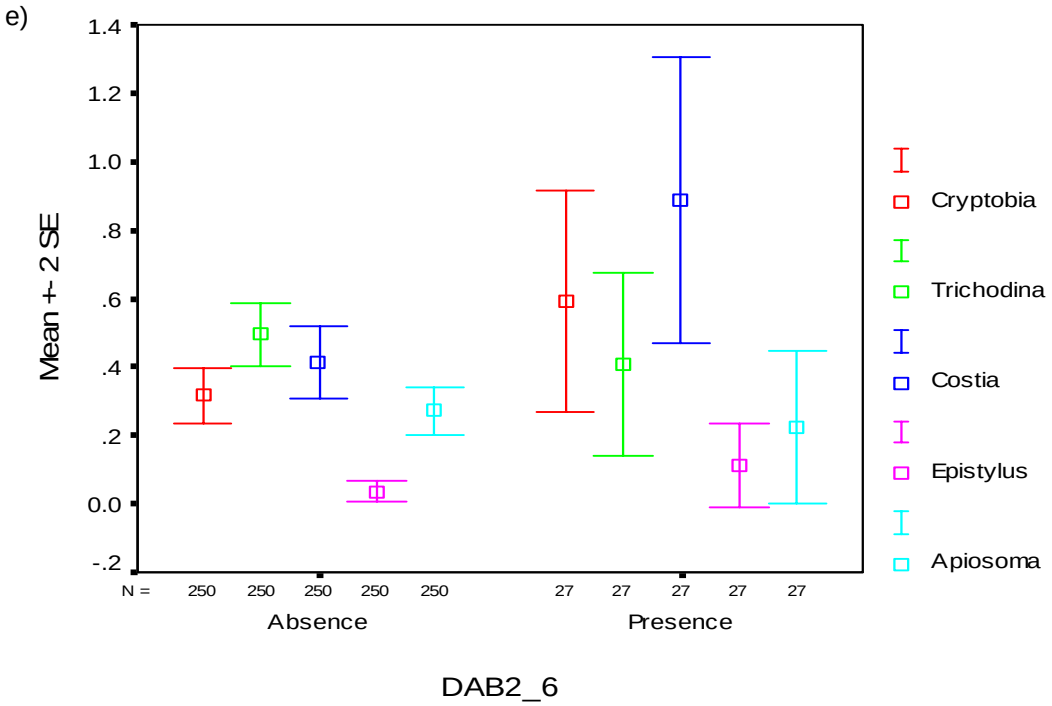


Figure 5 a-e. Standard error plots of mean parasite infection by Cyptobia, Trichodina, Costia, Epistylus and Apiosoma in the presence and absence of a) Hyam-DAB1*03, b) Hyam-DAB1*05,c) Hyam-DAB1*13, d) Hyam-DAB1(2)*04, e) Hyam-DAB1(2)*06.

to examine the relationship between this genetic variation and fish pathogen data. Major findings of this study include: i) identification of three evolutionarily divergent lineages of MHC Class IIβ alleles in Rio Grande silvery minnow; ii) differences in selective regimes between allelic groups, with evidence of strong selection in DAB1 and DAB1(2) groups; iii) retention of high levels of MHC Class IIβ allelic variation; iv) spatial differences in the amount of MHC Class IIβ diversity; v) no correlation between MHC Class IIβ and parasite diversity measures and vi) some evidence of association between particular alleles and presence or absence infection with particular pathogens. This study also includes the first description of MHC Class IIβ in a North American cyprinid fish and is one of only a handful of wild population-level studies that simultaneously compare MHC diversity and fish parasite communities.

Three divergent lineages of MHC Class IIβ alleles were identified in Rio Grande silvery minnow and two are highly homologous with allelic groups previously described in cyprinids (DAB1 and DAB3) (Ono *et al.* 1992; Graser *et al.* 1996; Sultmann *et al.* 1994; Ottova *et al.* 2005). The other allelic group [DAB1(2)] falls within the DAB1 group. Whether or not these lineages of alleles represent alleles of a single locus or are alleles of different loci is yet to be resolved (Ottova *et al.* 2005) and is complicated by the different ploidy (the number of homologous sets of chromosomes) levels in cyprinids. For example, the zebrafish and members of the Leuciscinae (includes North American cyprinids) are diploid (two sets of chromosomes), common carp is tetraploid (four sets of chromosomes) whilst the African 'large' barb is hexaploid (six sets of chromosomes). In the latter case, if there was a single heterozygous locus, the maximum number of alleles would be six (one allele per chromosome). With each additional locus, the number of alleles possible would double. The MHC sequences obtained during this study share homology with sections on zebrafish chromosome 8 (two distant locations) and to a single region on chromosome 13. DAB sequences identified in Rio Grande silvery minnow could therefore represent two paralagous sets of loci which has also been suggested for other cyprinid species (van Erp *et al.* 1996). Segregation studies performed in common carp suggested that there were two sets of paralagous loci (DAB1/DAB2 and DAB3/DAB4) that segregated independently (van Erp *et al.* 1996). Paralogous loci are clusters of genes that are at different chromosomal locations in the same organism but that are structurally similar indicating that they are derived from a common ancestral gene and have diverged following duplication or translocation by mutation, selection or drift. Additional data for Rio Grande silvery minnow exon 3 and

introns 2 and 3 (not presented) shows that exon 3 is highly divergent (between DAB1 and DAB3 alleles) while the introns cannot be aligned between these two allelic groups. This observation and the distant positions of DAB1 and DAB3 allelic groups in the phylogenetic tree, supports the notion that DAB1 and DAB3 may be distinct loci. The relationship between DAB1 and DAB1(2) is unclear, however the similarity of DAB1 and DAB1(2) exon 2, exon 3 and introns (data not included) suggest a more recent duplication event.

Selective regimes acting on each of the allelic lineages were variable which supports the possibility of multiple loci. There was evidence of positive, diversifying selection on all major groups of alleles including the Hyam-DAB3 group and the Hyam-DAB1 and Hyam-DAB1(2) groups with the majority of nucleotide substitutions resulting in amino acid changes. In several cases the d_N:d_S ratios were greater than three, a signature of strong positive selection. When the data was partitioned into sites thought to be involved with peptide binding and those that are non-peptide binding sites the results were more ambiguous. Hyam-DAB1 there was evidence for strong positive selection at peptide binding sites as well and non-peptide binding sites. For Hyam-DAB1(2) this was not the case and for Hyam-DAB3 only non-peptide binding sites appeared to be under positive selection. The designation of peptide binding sites in fish is based on the three dimensional structure of human HLA so it is possible that there are additional peptide binding sites in fish that are yet to be identified. Ottova *et al*. also considered the selective pressures acting on MHC allelic groups in Leucisinae and Cypininae and found evidence of positive selection on DAB3 for the former group, but on both DAB1 and DAB3 for the latter group. Ottova *et al.* (2007) suggests that DAB1 may have secondarily acquired function.

There are some puzzling features of Rio Grande silvery minnow DAB3 including the infrequency of transcription (and rarity of presence in genomic DNA) of DAB3 alleles (~10% of individuals) and the absence of any individuals with more than allele at this locus. A possible explanation for both of these observations is the presence of null DAB3 alleles at high frequencies in the population. A null allele is an allele that consistently fails to amplify and this may be because of mutations within the section of sequence where the PCR primer is designed to anneal. Alternatively there may be transcriptional regulation of expression whereby sequences are present in genomic DNA but not all are transcribed or alternatively, there may be inactivation of other MHC copies by codon deletions or accumulation of mutations, which would make the genes non-functional in

the immune response. The former has been demonstrated in polyploid species including the African 'large' barb (Kruiswijk *et al.* (2004), and the latter has been shown in the African clawed frog, *Xenopus* (Sato *et al.* 1993). We do not think that the former possibility is the case in Rio Grande silvery minnow, as DAB3 sequences from could not be amplified from the genomic DNA of any additional individuals. Van Erp *et al.* (1996) suggested that inheritance of MHC in cyprinids may not follow Mendelian rules.

We observed amino acid polymorphisms among alleles within the DAB3 group that were shared between divergent cyprinid lineages (e.g. Rio Grande silvery minnow, common carp and carp bream). These polymorphisms were often ones that distinguish DAB1 and DAB3 lineages. Sharing of residues may be a result of convergent evolution or may be a result of shared ancestry with maintenance of ancestral polymorphisms through selection (Hughes *et al.* 1994). In mammals, it has been suggested that there is an upper time limit (separations of 30-40 million years) for the sharing of ancestral MHC polymorphisms (Takahata & Nei 1990; Hughes *et al.* 1994). The family Cyprinidae is roughly 40 million years old (Zardoya & Doadrio 1999) and so is at the upper limit of the time scale suggested for conservation of shared polymorphism in mammals.

The Rio Grande silvery minnow population is characterized by high MHC Class IIβ diversity with 72 alleles recognized among 254 individuals. Taking the differences in sample sizes between studies into account, this diversity is comparable to the one cyprinid species for which limited population level data is available. The recent study of the carp bream identified 32 different exon 2 sequences (DAB1- 17 alleles, DAB3- 15 alleles) among 20 individuals (Ottova *et al.* 2007). A smaller dataset obtained for two species of zebrafish revealed 36 alleles (based on intron 1 and exon 2 sequences) from 28 fish (Graser *et al.* 1996). A similar comparison across four species of African 'large' barb recognized 29 DAB1 alleles among 16 individuals (Kruiswijk *et al.* 2005). Among salmonids, Dorschner *et al.* (2000) identified 42 MHC Class IIβ alleles among 74 individual lake trout and 88 alleles in steelhead trout (Aguilar & Garza 2006). However, high level of diversity appears to be the exception within this group with only nine alleles identified from 120 Atlantic salmon (Langefors 2001), 11 DAB1 alleles identified among 13,000 sockeye salmon and in Gila trout only five alleles were identified from 142 individuals assayed (Peters and Turner, In Press). Differences in habitat, environment and exposure to pathogens may account for differences in MHC diversity among cyprinid and salmonid taxa. For example, Rio Grande silvery minnow inhabits a warm-water system where parasites and pathogens are more prevalent than in cold-water

mountain streams. Unfortunately there is no MHC data from other Southwestern cyprinid taxa with which to compare our silvery minnow data, so we do not know if the level of MHC diversity in this species is 'normal'. In addition to large numbers of alleles, the level of divergence among DAB1 alleles is generally high in silvery minnow. Large divergence among alleles could be explained by either rapid mutation rate or long persistence time of allelic lineages (Klein *et al*. 1993). Findings of Grase et al. (1996) support the latter explanation.

The high level of MHC Class IIβ diversity seen in Rio Grande silvery minnow is in stark contrast to low levels of variation (in terms of number of alleles and the divergence between them) at the mtDNA ND4 locus. We have screened in excess of 3000 Rio Grande silvery minnow for mtDNA ND4 variation and only identified 14 closely related ND4 alleles (Osborne et al. 2005). The low level of mtDNA diversity is indicative of a severe historical population bottleneck in Rio Grande silvery minnow. Furthermore the contemporary population is characterized by a small genetic effective size (N_{ev}~100 Turner and Osborne unpublished data). Differences in levels of variability between the different markers (mtDNA, microsatellites and MHC) are likely due to differences in selective pressures, ploidy levels, mutation rates and inheritance patterns.

An unexpected result of this study was the difference in the number of MHC Class IIβ alleles expressed at each of the localities, with fewer alleles expressed by fish in southern sites in 2006. In 2008 however there were similar numbers of MHC alleles detected at each of the localities with the highest number seen at La Joya. Data from neutral markers (microsatellites and mtDNA) allow genetic drift to be discounted as a driving factor behind this observation, as we see no differences in number of alleles identified at these loci. In addition, frequencies of mtDNA-ND4 haplotypes and microsatellite alleles do not differ significantly among collection localities, which is consistent with high gene flow between the Rio Grande silvery minnow sampling localities (Osborne *et al.* 2005). Rio Grande silvery minnow produce non-adhesive pelagic eggs that are subject to drift with river currents (Platania and Altenbach 1998, Osborne *et al.* 2005) allowing homogenization of the population. Although there are water diversion structures that preclude upstream movement, management activities, particularly augmentation of the wild population with captively reared fish, also results in mixing of the population.

There have only been a handful of studies that have considered the relationship between MHC and pathogens in fish. From these studies, three conclusions have been

drawn: i) survival to infection and susceptibility may be associated with specific MHC alleles (e.g. Langefors 2001); ii) resistance or survival from infection may be associated with increased MHC heterozygosity (Arkush *et al.* 2002; Hedrick *et al.* 2001b) or; iii) parasites may drive MHC variability but there is an optimal number of MHC alleles within an individual (Wegner *et al.* 2003). In Rio Grande silvery minnow we were only able to test for a relationship between the number of MHC alleles and number of allelic groups per fish as heterozygosity and nucleotide diversity could not be calculated (because alleles could not assigned to loci). A significant association between the number of MHC Class IIβ alleles expressed and the number of parasites infecting an individual was not seen. There was also no relationship observed between the number of allelic groups represented (within individuals) and parasite diversity measures (number of species and overall level of infection). This is concordant with the results of Ottova (2007). In fact, in carp bream, expression of two alleles from a single allelic type was associated with higher ecto-parasite loads (Ottova *et al.* 2007). They also found that increased nucleotide diversity measured at alleles of a single type was associated with higher abundances of the ecto-parasites *Dactylogyrus* spp. and *Ergasilus* spp., and that immunocompetance was lower in these fish.

Although not rigorously tested statistically (because of relatively small sample sizes), there did appear to be an association between expression of Hyam-DAB1*05 by Rio Grande silvery minnow and lighter infection by *Costia* spp. There also appeared to be an association between the presence of Hyam-DAB1(2)*06 and heavier *Costia* spp. Infection and between presence of Hyam-DAB1*13 and heavier *Apiosoma* infection. If these relationships prove robust it would be the first report in a cyprinid fish of a specific association between an MHC allele and a parasite species. In carp bream no association was detected between expression of certain MHC Class IIβ alleles and resistance to specific parasites (Ottova *et al.* 2007). This was also true for the three-spined stickleback (*Gasterosteus aculeatus*) (Wegner *et al.* 2003). These species are characterized by large numbers of alleles that are generally present at low frequencies within the population. This combination of factors limits the power to test for correlations between parasite species richness and abundance and MHC diversity. Rio Grande silvery minnow MHC Class IIβ also has many alleles but most are present at low, so it is surprising that we see evidence of relationships between particular alleles and infection with particular parasites. There may also be effects of collection locality on these results.

In contrast to cyprinids, salmonids have a single MHC Class IIβ locus and few alleles present at high frequencies improving chances of detecting significant associations (Stet *et al.* 2002). The salmonid studies also selected particular pathogens *a priori* to test for association with MHC alleles (Miller *et al.* 2004; Grimholdt et al 2003). The ecto-parasites that were encountered on Rio Grande silvery minnow gills were all generalist, opportunistic pathogens that have direct life-cycles (i.e. no intermediate host). 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 and hence MHC polymorphism should be more impacted by specific parasites. The relationship between the hosts immune system and parasites is also likely to be a dynamic one and immunity/susceptibility may also involve interaction between multiple alleles.

The relationship of parasites, hosts and their variation at MHC is determined by a long list of factors that include both biotic and abiotic features that are intertwined. Some of the factors that affect parasite abundance include the physiology and biological features of the host, chemical properties of the water, the age of the fish (and hence its size), diet of the host, size and connectivity of the habitat that the fish occupies (reviewed in Dogiel *et al.* 1961) and the history of co-evolution between host and pathogen. MHC is not only involved with immunity but in some species of fish these genes play a role in mate choice so there may be a tradeoff between these functions. The present study on MHC Class IIβ genes within the wild population of Rio Grande silvery minnow provides a crucial data set that will allow future investigations of the various factors that may drive diversity at MHC genes of this species.

Conclusions and Implications for Management

MHC variation in in Rio Grande silvery minnow is decoupled from microsatellite and ND4 variation. Further investigation of MHC Class IIβ genes in Rio Grande silvery minnow is required to understand the mechanisms that drive diversity at these genes.

Further research will involve trying to determine the number of MHC loci that are present in this species. This will allow more accurate measures of MHC diversity to be obtained. Coupling this information with increased sample size and more accurate measures of parasite load would improve our power to detect associations between MHC and parasite diversity. Variation in MHC allele frequencies among populations and the high number of rare MHC alleles argues for wide collection of broodstock for hatchery practices, or ideally collection of wild-caught eggs encompassing the geographic range

of the species. Further research is needed that seeks to understand whether there is: i) transcriptional regulated of allelic variation or; ii) regulation at the genomic level which would allow a deeper understanding of management implications of MHC variation in Rio Grande silvery minnow.

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