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Functional implications of Major Histocompatibility (MH) variation using estuarine fish populations

Sarah Cohen,[1,*] Joëlle Tirindelli,* Marta Gomez-Chiarri,[†] and Diane Nacci[§]

*Romberg Tiburon Center, Department of Biology, San Francisco State University, 3152 Paradise Dr., Tiburon, CA 94920-1205, USA; [†]Department of Fisheries, Animal and Veterinary Science, University of Rhode Island, Kingston, RI 02881, USA; and [§]US Environmental Protection Agency, Office of Research and Development, Atlantic Ecology Division, Narragansett, RI 02882, USA

Synopsis Recently, there has been a dramatic expansion of studies of major histocompatibility complex (MHC) variation aimed at discovering functional differences in immunity across wild populations of diverse vertebrate species. Some species with relatively low genetic diversity or under strong directional selection by pathogens have revealed fascinating cases of MHC allelic disease linkage. More generally in genetically diverse species, however, these linkages may be hard to find. In this paper, we review approaches for assessing functional variation in MHC and discuss their potential use for discovering smaller-scale intraspecific spatial and temporal patterns of MHC variation. Then, we describe and illustrate an approach using the structural model to produce a population composite of variation in antigen-binding regions by mapping population-specific substitutions onto functional regions of the molecule. We are producing models of variation in major histocompatibility (MH) loci for populations of non-migratory fish (killifish, *Fundulus heteroclitus*) resident at sites that vary dramatically in environmental quality. We discuss the goal of relating MH population variation to functional differences in disease susceptibility such as those inferred by observations of parasitic infection and direct measurement of bacterial challenges in the laboratory. Our study has focused on relatively well-studied killifish populations, including those resident in a highly disturbed, chemically contaminated estuary and nearby less contaminated sites. Population-specific genetic changes at MHC antigen-binding loci are described, and evidence relevant to functional implications of these changes is reviewed. Population-specific patterns of variation in antigen-binding regions in combination with a range of assessments of immune function will provide a powerful new approach to reveal functional changes in MHC.

Introduction

The major histocompatibility complex (MHC in most vertebrates or MH in bony fish) (Stet and others 2003) is receiving increasing attention from ecologists and evolutionary biologists interested in the dynamics of this highly variable genetic system in natural populations (Hess and Edwards 2002; Bernatchez and Landry 2003; Sommer 2005). Furthermore, both the impact and mode of immunogenetic selection on diversity in wild species is being addressed now as researchers attempt to tease apart high levels of polymorphism that are indicative of past versus recent selective pressures (for example, Charbonnel and Pemberton 2005). The MHC processes and binds antigens from pathogens and presents these antigens to the adaptive immune system, playing an essential role in the vertebrate immune response mechanism (Parham and Ohta 1996; Edwards and Hedrick 1998; Hess and Edwards 2002). MH class I genes are ubiquitously expressed on nucleated cell surfaces where they bind peptides derived from intracellular pathogens such as viruses. In contrast, class II genes have a more limited tissue distribution and function in the binding of antigens derived from extracellular pathogens such as bacterial invaders and flatworms, for example (Hess and Edwards 2002; Sommer 2005). Positive selection, which is selection that favors novel variants (Nielsen 2005), is thought to maintain the extremely high polymorphism observed in MHC genes at the population level, since increased variation in MHC would increase the suite of pathogens that an organism is able to combat (Klein and others 1993; Edwards and Hedrick 1998; Hess and Edwards 2002). In particular, the study of MHC class II genes in wild vertebrate populations (from fish to mammals) has provided valuable insight into local adaptation, evolutionary processes, and functional differences found within and between natural populations that

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[1] E-mail: sarahcoh@sfsu.edu

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have advanced conservation biology (Edwards and Hedrick 1998; Bernatchez and Landry 2003; Sommer 2005). Class II genes are the most highly variable MHC loci found to date in most taxa and have been used the most in wild population studies.

A major reason for the initial use of the MHC at the intraspecific level in non-model organisms was the need for highly variable markers to differentiate populations under severe demographic stress, where most available genetic markers showed little or no variation. An additional driving force in the expansion of MHC studies in non-model systems has been the increasing accessibility of molecular techniques for assaying genetic variation in the nuclear genome. Standard techniques now used in the laboratories of some ecologists and evolutionary biologists include cloning, sequencing, single-strand conformation polymorphism (SSCP), and denaturing gel gradient electrophoresis (DGGE) as well as the development and use of MHC primers for non-model taxa. Accompanying the development of more accessible methods for obtaining MHC data from novel species is a parallel development of computational methods including increasingly sophisticated molecular evolutionary tools for testing selective hypotheses (focus of first section of this review). This rapid development and portability of bioinformatics tools to non-model systems has widened opportunities for testing how extraordinary MHC diversity is generated and maintained in wild populations (Bernatchez and Landry 2003). Thus, MHC studies of wild populations have moved from tests for diversity in bottlenecked populations to a broader realm using more complicated tests for impacts of selection in non-constricted populations. Accompanying this shift in techniques and in the species used in MHC studies is a more recent focus on shorter-term signals of selection on the MHC, testing for responsiveness of populations to local selective pressures thought to change over shorter spatial and temporal scales in comparison to the longer term, often transpecific focus of early MHC studies.

An important feature of MHC studies in wild populations is the opportunity to take advantage of the inferred functional importance of the remaining immunogenetic variation, and evaluate its presumptive role in responding to antigenic challenge. Evaluation of the functional consequences of variation in MHC provides a path to the study of the ecological and evolutionary mechanisms that generate and maintain MHC diversity in natural populations [reviewed most recently by Piertney and Oliver (2006)]. Recently, an increasing number of studies in mammals related temporal patterns of parasitism, as a phenotypic measure of the functional consequences of variation in MHC, to MHC genotypes. One impressive long-term study of immunogenetic variation in response to environmental cycling in a semi-natural population is the Soay sheep example, in which Mhc-linked microsatellite allele frequencies cycle in correlation with parasite populations, which are in turn intriguingly linked to climate variability in the form of fluctuation in rainfall (Paterson and others 1998; reviewed by O'Brien 2000).

Spatio-temporal information on how variation in selective forces, for example, parasite population dynamics, and disease pressure, is reflected in MHC diversity at local levels can be challenging to obtain. Variation in selective forces and very high levels of allelic diversity in MHC genes (Piertney and Oliver 2006) may obscure obvious allelic linkages. There is a scarcity of studies of MHC in natural populations that demonstrate associations between allele frequency and parameters such as disease or parasite prevalence, given that the MHC plays a critical role in the adaptive immune system. One reason for this scarcity is that often in MHC studies, allele frequency analysis is hampered by the low sample sizes of MHC alleles that are a consequence of high diversity of MHC, and of balancing selection (Hill 1999; Schad and others 2005; Sommer 2005). To date, in MH studies of fish, background levels of genetic diversity vary widely (Table 1). In several cases (salmonid species), allelic disease or parasitic linkages have been discovered in very low MH-diversity systems where bottlenecking may have occurred, but power to detect associations is high. In other fish species (killifish, sticklebacks), however, high genetic variation makes it more difficult to tease out signals of selection from the overwhelming level of background variation. In fact, there is sometimes little or no overlap in MH alleles sampled across populations. For example, only 1–2 shared MH class II alleles out of a total of 41 alleles were found in studies of wild Atlantic killifish (Cohen 2002) and Atlantic salmon (Consuegra and others 2005) populations.

Here, we present work on developing immunogenetic tools for studying MHC in wild populations, and our progress in applying these to wild, highly genetically diverse estuarine killifish (*Fundulus heteroclitus*) populations under varying degrees and types of environmental stress (selective pressures). These immunogenetic tools take advantage of the functional structural model to detect signals of selection in populations with highly diverse MH loci. Moreover, we also wish to use our killifish example to highlight some diverse and complementary approaches for assaying function of the immune system function in wild

Table 1 Diversity of MH IIDB in wild populations of fishes

Fish species	MH IIDB exon 2 sequence length	Distinct DNA alleles/individuals sampled	Ratio and % polymorphic amino acid sites	Reference
Atlantic killifish (*Fundulus heteroclitus*)	Lacking 14 bp 3' (254 bp)	41/35 1.17	52/84 (61.9%)	Cohen (2002)
Stickleback (*Gasterosteus aculeatus*)	Partial (210 bp)	31/48 0.65[a]	36/70 (51.4%)[b]	Reusch and Langefors (2005)
Lake whitefish (*Coregonus sp.*)	Partial[c] (249–252 bp)	20/15 1.33	32/84 (38%)	Binz and others (2001)
Gila topminnow (*Poeciliopsis o. occidentalis*)	Partial (188 bp)	17/13 1.31[a]	22/62 (35.4%)[b]	Hedrick and others (2001)
California coastal steelhead (*Oncorhynchus mykiss*)	Partial (216 bp)	88/444 0.20[a]	—	Aguilar and Garza (2006)
Atlantic salmon (*Salmo salar*)	Partial[c] (254 bp)	18/666 0.03[a]	23/84 (27.4%)[b]	Landry and Bernatchez (2001)
Sockeye salmon (*Oncorhynchus nerka*)	Partial (216 bp)	11/5400 <0.00[d]	10/72 (14%)	Miller and others (2001)
Chinook salmon (*Oncorhynchus tshawytscha*)	Partial[c] (260 bp)	6/175 0.03[a]	7/65 (10.8%)	Kim and others (1999)

Some of the variation shown may be due to differences in sampling or in genetic methods. See individual references for more information on the methods and on the MH IIDB polymorphism for each species.
[a]SSCP was used to identify allelic variants.
[b]Number of variable amino acid sites manually counted from published alignment.
[c]Established based on lack of one or more PBR sites (Brown and others 1993) in amino acid alignment.
[d]DGGE was used to identify allelic variants.

populations. Furthermore, in addition to tracking population dynamics of parasites in relation to MHC variation, there are other opportunities for phenotypic measures of immune system function for diverse ecological and evolutionary questions (for example, invasive species) (reviewed by Lee and Klasing 2004).

Application of the functional structural MHC model

The 3D structure of human class II MHC molecules and a functional model of peptide binding were first established by Brown and others (1993) and Stern and others (1994). X-ray crystallography of human MHC molecules with bound peptides was used to determine the 3D structure and the sites involved in peptide binding in these studies. The functional model describes the peptide-binding region (PBR) and the amino acid codons found in the PBR on the protein tertiary structure. Most MHC class II studies take advantage of the available functional model, but to varying degrees.

In the next sections, we review the techniques available for studying genetic variation in MHC genes that make extensive use of the functional model and discuss their current or possible application to MHC studies in natural populations. Our focus is mainly on the current, or potential, application of these techniques to the study of the highly polymorphic portion of the MH class II binding cleft, the beta-chain (β-chain), in investigating recent, local adaptation among natural populations. We present our own application of certain techniques to the study of genetic variation in the MH class IIDB, which are MH class II β-chain loci, in an estuarine killifish, *F. heteroclitus*.

Traditional dN/dS test of selection

A common application of the functional model has been to narrow substitution-rate analysis, dN/dS ratio, to PBR sites, to test for positive selection at these codons. The dN/dS ratio test of selection compares the rates of non-synonymous (dN) and synonymous, or silent (dS), substitutions. In the absence of any selection, the rate of non-synonymous substitutions, dN, should equal the rate of synonymous substitutions, dS. A dN to dS ratio greater than 1 indicates positive selection, or selection favoring new variants (Hughes and Nei 1989).

In the majority of studies conducted on non-model taxa, dN has been shown to be significantly greater than dS in the PBR, but not in other parts of the MHC gene (Bernatchez and Landry 2003). While the traditional dN/dS ratio test has been pivotal in providing evidence for positive selection in MHC genes, it is a very conservative measure (Nielsen 2005). One reason that the dN/dS ratio test is a conservative measure is that a proportion of non-synonymous

substitutions may be experiencing negative selection in order to preserve structurally related function (even at PBR sites) (Pakula and Sauer 1989; Tang and others 2004; Tang and Wu 2006). The rate of non-synonymous substitutions, dN, is then likely to be underestimated due to the mixed signal of negative and positive selection at non-synonymous sites (Tang and Wu 2006).

Recent developments in selection tests

The recent developments proposed and discussed below address the complexity in estimates of dN and dS and aim to refine substitution-rate analysis for detecting positive selection. One widely applied, new development of the dN/dS test uses a likelihood-ratio method to test different models of substitution using a phylogenetic tree (Yang and Bielawski 2000; Yang and others 2000; Yang 2002). Some of the models incorporate a higher rate of non-synonymous than synonymous substitutions at some sites, and therefore indicate positive selection. The major advantages of this technique are that it is able to allow for different intensities of selection along the sequence instead of simply averaging across sites. For examples of the implementation of this method in wild populations, see Bos and DeWoody (2005), Consuegra and others (2005) and Schaschl and others (2005). The model of substitution designated by the maximum-likelihood analysis can also be used in subsequent Bayesian analysis to infer which specific residues are under positive selection (Yang and others 2005). This posterior Bayesian analysis has the potential to provide a test for differential selection at specific codons among populations and may be very useful for testing theories of population-level adaptation that are predicted to be reflected by this type of variation of MHC.

The applicability of the methods of Yang and others (2000, 2002, 2005) to MHC studies in natural populations is impeded by the fact that it is prone to inaccuracy at high rates of recombination (Anisimova and others 2003). Varying levels of recombination have been shown for MHC studies in natural populations, with measures of recombination rate, ρ, of 10–11 and 14–25 for Irish and Norwegian Atlantic salmon populations, respectively (Consuegra and others 2005), 37 and 78 for Pyrenean and Alpine chamois, respectively (Schaschl and others 2005), 88 for voles (Bryja and others 2006) and $\geq$100 in 3-spined sticklebacks (Reusch and Langefors 2005) and *F. heteroclitus* (S Cohen unpublished data). For approximate guidelines for the level of recombination likely to have an effect on this test of positive selection, Anisimova and others (2003) suggest fewer than 3 recombinant events in a sample of 10 sequences will provide accurate estimates.

The next 2 types of positive tests for selection presented here are similar in that they divide non-synonymous substitutions, or amino acid changes, into various categories. Non-synonymous amino acid substitutions have long been categorized by either the rate in which they substitute for one another (for example the BLOSUM matrices) (Henikoff and Henikoff 1992) or by their similarity in certain physicochemical properties (Grantham 1974). The method of non-synonymous substitution categorization for measuring rates of amino acid substitutions developed by Tang and others (2004) and Tang and Wu (2006) separates highly exchangeable amino acids from other amino acid substitutions. As opposed to dN, which includes all non-synonymous substitutions, Tang and Wu (2006) distinguished rates of high versus low-exchangeability substitutions as a way to correct for underestimation of dN in the traditional dN/dS test, discussed earlier. A ratio of Kh (the rate of substitution of highly exchangeable amino acids) to Ks (equivalent to dS) that is significantly greater than 1 is considered evidence for positive selection (Tang and Wu 2006). Highly exchangeable amino acids are defined by empirical data collected from many genes and across a wide range of taxa using a codon-based approach (Tang and others 2004).

While Tang and Wu (2006) described their classification system as being universally applicable, given the distinctive mode of evolution of MHC genes, where low-exchangeability amino acids may be favored when they significantly alter antigen-binding abilities while not disrupting overall function, the frequency of certain types of amino acid changes is likely to be different from those of other genes. For MHC analysis, the method proposed by Tang and Wu (2006) could be greatly enhanced by the development of a separate categorization system based on empirical data from MHC genes alone.

In the method developed by Sainudiin and others (2005), the non-synonymous and synonymous categories are replaced by categories of property-altering and property-conserving substitutions, respectively. The rate of amino acid substitutions that alter charge alone, polarity alone, polarity and/or volume and volume alone can be tested and compared to the rate of property-conserving substitutions (Sainudiin and others 2005). The method developed by Sainudiin and others (2005) incorporates the maximum-likelihood test of Yang and others (2000) and offers a posterior Bayesian approach similar to that described in Yang and others (2005) to infer the residues under property-altering positive selection for each physicochemical

property. The categories of properties used in this method were chosen because of the important role they play in the interactions between receptor and ligand; the technique was developed using a human Mhc class I example; therefore, the method should appropriately detect the selective forces on MHC genes. This method, however, relies on the phylogeny-based maximum-likelihood approach of Yang and others (2005) and, likewise, is hampered by high rates of recombination (Anisimova and others 2003; Sainudiin and others 2005).

Allele supertype frequency

A novel method of pooling alleles into a smaller number of functional categories, MHC "supertypes," may increase statistical power in frequency-based analyses (Trachtenberg and others 2003; Sommer 2005). In this technique, alleles are grouped into supertype categories that reflect common binding function (Southwood and others 1998; Sette and Sidney 1999; Doytchinova and others 2004; Doytchinova and Flower 2005). The frequencies of pooled alleles that are functionally similar can then be compared, resulting in increased power to detect associations between MHC functional alleles and disease.

Trachtenberg and others (2003) applied the supertype frequency method to a study of MHC class I in humans and found associations between certain supertypes and HIV progression. The supertype classification they used was developed by Sette and Sidney (1999), who used empirical data on the repertoire of peptides known to bind to different MHC molecules to develop their supertype categorization. Within supertypes, Sette and Sidney (1999) identified similarities in the patterns of peptide-binding codons that cluster together into regions on the 3-dimensional structure of MHC molecules, known as peptide-binding pockets. Many human MHC class I and class II supertype classification schemes have relied on peptide-binding pocket motifs as "fingerprints" to predict peptide binding and the categorization of alleles into supertypes (Chelvanayagam 1997; Ou and others 1998; Doytchinova and Flower 2005).

Supertype methods are a promising avenue for MHC studies in natural populations, since they have the potential of allowing researchers to test for correlations among groups of functionally similar MHC alleles and parameters such as disease or pathogen prevalence. It appears, however, that more research must be done (for example, developing bioinformatics tools and performing peptide-binding assays either *in vitro* or *in silico*) before MHC class II allele supertype categorization and frequency analysis may be applied to a wide range of non-human taxa. For instance, many of the amino acids at certain PBR sites that are considered "fingerprints" for the supertype classification of human MHC class II alleles provided by Doytchinova and Flower (2005) were not found in approximately 100 distinct amino acid alleles sampled from 5 wild populations of the Atlantic killifish, *F. heteroclitus* (Cohen 2002; J Tirindelli unpublished data). It is possible, however, that the currently available MHC human class II supertype classifications are applicable to the study of wild populations of more closely related taxa, such as other primates.

Amino acid composition analysis

The main principle behind supertype categorization is the use of amino acid substitutions at PBR sites as "fingerprints" of peptide-binding function. This principle can be applied to MHC studies in wild populations, regardless of the state of supertype development for non-human taxa. Studies of MHC class I and class II peptide binding have shown that 1 to 3 amino acid substitutions are sufficient to influence binding function (Davenport and others 1995; Jurcevic and others 1996; Diab and others 1999; Carroll and others 2002), which affects the adaptive immune response. These studies demonstrate the use of amino acid substitution patterns in finding signals of pathogen-mediated selection acting on MHC genes in natural populations. As with dN/dS ratios, the study of MHC class II amino acid composition can be narrowed by focusing on the peptide-binding region (PBR) sites that are defined according to the functional model first proposed by Brown and others (1993) and Stern and others (1994).

Several methods have been used to apply the information in the crystal structure and functional antigen-binding model to look for significant differences at the population level. Some analyses have compared substitution differences between populations by looking only at unique population-specific substitutions while others have considered frequency differences in substitution patterns between populations. In addition, some studies have made use of any population differences in substitution patterns while others have specifically considered either PBR sites or particular binding pockets (a subset of all PBR sites) and their associated codons. In the folded 3-dimensional structure of the MHC molecule, particular codons, which are often non-adjacent in linear sequence, cluster together in 1 or more of 5 peptide-binding pockets, referred to as pockets 1, 4,

6, 7, and 9 (Stern and others 1994). Peptide-binding pockets, as mentioned in the previous section, are cup-shaped areas of the molecule that are important in peptide binding (Stern and others 1994) and, therefore, in pathogen recognition.

An example of the usefulness of examining amino acid composition for MHC studies in natural populations is given by a study of intraspecific variation in MHC in the Malagasy mouse lemur by Schad and others (2005). The authors' comparison of variation in amino acid substitution resulted in the finding of unique amino acids at PBR sites in one MHC allele significantly associated with parasite-infected lemurs and in 2 alleles significantly associated with uninfected lemurs. The Malagasy mouse lemur appears to show high MHC class II DB diversity (56% of amino acid sites were variable, specifically at the DRB locus), although the number of distinct alleles found (by SSCP) per individuals sampled was low (2/83 or 0.02) (Schad and others 2005).

Comparing amino acid substitutions at MHC loci, and particularly at sites thought to be important in peptide binding, provides a focus on functionally relevant MHC variation. This is an especially useful approach for studies of MHC class IIDB variation in species demonstrating high genetic variation, where a signal of selection may be otherwise difficult to tease out of the overwhelming level of background variation (Cohen 2002). Thus, it is in the more genetically diverse species and populations, that use of the functional structural MHC model and more specific techniques for functional analysis are mandated. Our analyses of MHC IIDB amino acid substitution variation in Atlantic killifish populations experiencing different environmental and pathogenic pressures are provided in subsequent sections.

Populations of *F. heteroclitus*

Fundulus heteroclitus has a broad geographic range (Atlantic Canada to the Gulf of Mexico) and large homeostatic ranges for temperature, salinity, and oxygen level. In general, individual fish are non-migratory estuarine residents predicted to have home ranges limited by the availability of local patches of habitat (Brown and Chapman 1991). As a result, rates of migration between populations and estuaries are relatively low (but generally sufficient to minimize genetic drift between adjacent estuaries) (Adams and others 2006). Broad distributional ranges and large, rather isolated non-migratory populations have contributed to the characterization of *F. heteroclitus* as comprising locally adapted populations (Mitton and Koehn 1975; Mitton 1997). These demographic attributes have made this species an important model for the exploration of mechanisms of adaptation and natural selection (for example, reviewed by Mitton 1997). In addition, their hardiness, small size, abundance, ease of collection and maintenance, and their suitability for manipulation in laboratory settings have made *F. heteroclitus* a model organism in experimental biology and physiology since the late 1800s (Powers 1989). These features make *Fundulus* an ideal model for investigating the functional implications of local genetic variation in MHC.

As part of a larger project to characterize population-level responses to multi-generational exposures to stressors, we are studying *F. heteroclitus* populations residing along the Atlantic coast of the United States. Populations have been sampled from sites that vary widely in environmental and pathogenic pressures, that is, pristine, residential, and urban/industrial estuarine sites. These sites vary by many orders of magnitude in chemical contamination, as indicated by concentrations of polychlorinated biphenyls (PCBs) in sediments (Nacci, Champlin and others 2002). We have used this metric of anthropogenic stress because these contaminants are widespread indicators of a constellation of contaminants and pathogens associated with human activities. We are also interested in PCBs because some congeners in this class act like the very toxic contaminant, dioxin (and are therefore categorized as "dioxin-like compounds," or DLCs), whose impact and mechanism of action has been a subject of much study.

One of the most highly contaminated and most well-studied sites from which resident populations of *F. heteroclitus* have been collected is New Bedford Harbor (NBH), Massachussetts. NBH is a highly disturbed estuary, designated by the United States Environmental Protection Agency as a Superfund site because of extremely high concentrations of PCBs. *F. heteroclitus* from NBH are highly contaminated with PCBs at levels that are known to produce acutely toxic effects (for example, lethality during sensitive early life stages (Nacci and others 1999; Nacci, Champlin and others 2002), as well as sublethal effects, including reproductive impairment (Black and others 1998; Gutjahr-Gobell and others 1999) in populations resident in less contaminated sites. Thus, laboratory studies have clearly demonstrated that *F. heteroclitus* from NBH have an adaptive phenotype, that is, an inherited and profound tolerance to some of the toxic effects of PCBs (Nacci and others 1999; Nacci, Champlin and others 2002). Furthermore, the presence of abundant and persistent field populations throughout NBH supports the conclusion of evolved tolerance (reviewed by Nacci, Gleason and others 2002).

The occurrence and maintenance of adaptive phenotypes in the NBH population and the known history of chemical contamination at the site (for example, Nelson and others 1996; Nacci, Gleason and others 2002) suggested that despite potentially high gene flow strong divergence has occurred in response to intense and recent (Post 1940) chemical contamination. Complementary approaches have been used to investigate genetic mechanisms and potential consequences of this rapid adaptation. For example, neither amplified fragment-length polymorphisms (McMillan and others 2006), allozymes (Roark and others 2005), the hypervariable I region of the mitochondrial control region (Cohen 2002) nor microsatellite loci in a somewhat limited population sample ($n = 20$) (Adams and others 2006) suggest reproductive isolation or genetic erosion in NBH fish. Sequence variation in genetic loci mechanistically associated with chemical tolerance (for example, in the aryl hydrocarbon receptor pathway) are also under investigation in chemically tolerant populations of *F. heteroclitus* from NBH and other locations (Karchner and others 1999; Yang 2003; Hahn and others 2004; S Cohen, G Yang, S Karchner, and M Hahn unpublished data). Comparison of sequence data from a portion of the transactivation region of the aryl hydrocarbon receptor (AHR1) from populations ranging from New Hampshire to South Carolina showed a significant relationship between haplotype frequencies and level of DLC contamination in the approximately 20 estuaries sampled (S Cohen, G Yang, S-M Tam, D Champlin, and D Nacci unpublished data).

In addition, the extreme contamination and degraded condition of NBH do have some discernible physiological impacts on *F. heteroclitus*. For example, NBH *F. heteroclitus* demonstrate evidence of exposure to endocrine disrupting chemicals, such as PCB metabolites, but also potentially including many other anthropogenic contaminants (Greytak and others 2005). Long-term exposure to high concentrations of PCBs could also have a significant impact on the immune system. Short-term exposure to high levels of PCBs and other chemical contaminants is known to contribute to increased susceptibility to disease in some fish species (for example, Loge and others 2005). Consistent with the expectation of immunosuppression due to exposure to pollutants, NBH *Fundulus* show unusual and extreme examples of parasitism in comparison to nearby reference populations (Table 2). Higher prevalences of metacercaria of *Ascocotyle tenuicollis* were consistently detected in the hearts of fish from NBH in comparison to those from a variety of near and distant reference populations. Furthermore, the adult stage of *Stomaticolla rubea* was detected in unusually high levels in the swim bladder of killifish from NBH in 1998 and 1999, but not in reference populations. In addition, sampling of additional sites in Pt Judith, RI, Gloucester, MA, Beaufort, NC, and Georgetown, SC failed to discover either parasite in killifish. Altered parasite communities in contaminated sites such as NBH may also result from other changes in community structure such as the composition of available host species or variable tolerance of parasites to contaminants. Neither the exact mechanism by which NBH *F. heteroclitus* tolerate PCBs (for review see Hahn 1998; Van Veld and Nacci 2006) nor the effect of long-term exposure to PCBs on susceptibility to disease in fishes are well understood (for example, Schmalz and others 2002).

The characteristics of *Fundulus* populations (including large population sizes, high levels of genetic variation, and limited dispersal) that permit them to adapt to environmental factors and anthropogenic stressors make these populations uniquely appropriate to study the mechanisms that generate and maintain MHC diversity in natural populations (Piertney and Oliver 2006). Our laboratories have joined efforts to characterize these *Fundulus* populations at ecological, physiological, immunological, and genetic levels.

Patterns of MHC variation in Fundulus populations

While the MHC has often been thought to reflect responses to ancient selective signals, even across

Table 2 Prevalence (number of fish with parasite/total number of fish sampled) and percent prevalence in parenthesis of two major parasites (digenetic trematodes) of killifish in polluted New Bedford Harbor (NBH) and the reference populations of Slocum's Island (SL) and West Island (WI), Massachusetts

	Sampling date and location					
	Aug 98	June–September 99			June 02	December 02
	NBH	SL	WI	NBH	NBH	NBH
Ascocotyle tenuicollis	ND	23/46 (50%)	1/10 (10%)	38/38 (100%)	25/25 (100%)	29/30 (97%)
Stomaticolla rubea	16/20 (80%)	0/46 (0%)	0/10 (0%)	10/38 (26%)	0/25 (0%)	0/30 (0%)

Parasite data from Cohen (2000), Hicks and Steele (2003), and M Huber and S Cohen (unpublished data). Identifications of parasites courtesy of R Overstreet.

species boundaries, there are abundant examples of the effects of more recent selection as well. These include human examples (for example, malaria Hill and others 1991) and HIV (Carrington 1999) in a system of relatively low genetic diversity as well as in higher diversity systems, such as stickleback fish (Wegner and others 2003). In high-diversity systems, specific allelic correlations, at either the DNA or amino acid levels, are hard to detect, perhaps contributing to the idea that local signals are not visible. In such cases, alternative, more functionally directed analyses using the structural antigen-binding model are necessary, as discussed above. In our analyses of patterns of MH class II DB substitution in divergent *Fundulus* populations, we have found that the use of a diversity of approaches, based on application of the structural model, is important for discovering population-specific patterns and signatures of differentiation.

In a study focused on NBH and associated reference populations, Cohen (2002) examined DNA variation and amino acid substitutions at MH II DB antigen-binding loci to test whether intense selection in a chemically contaminated environment would produce a signature of local adaptation in the immune system or in other traits outlined above. Selective scenarios related to chemical contamination include direct (although largely unknown; see above) effects of xenobiotic compounds on immune receptors and system function. More obviously, however, contaminated environments such as NBH house significantly altered parasite communities (Table 2). These altered communities then produce strong differences in the suites of antigens challenging the MHC of resident species.

As in most *F. heteroclitus* populations, genetic diversity at MH loci in NBH and in fish from reference populations was high and the dN/dS ratio at PBR, but not other amino acid sites, was significantly greater than 1. Alleles from genomic samples were not obviously lacking function (for example, due to missense mutations). Thus, the MH class 2 loci appear functional by these rather basic measures of population genetics and molecular evolution. In addition, features of the innate immune system as measured in acute laboratory bacterial challenges (discussed in a later section of this review) also showed functionality in NBH and in fish from reference sites.

Strong differences in MH class 2 substitution patterns were found in NBH fish relative to fish from reference sites when population-specific amino acid replacements were mapped on the structural molecular model. In a comparison of NBH with the reference site at Anisquam Inlet of Gloucester, Massachusetts, signature amino acid substitutions from NBH are significantly focused in pocket 7 whereas population-specific substitutions for Gloucester are focused in binding pocket 6 (see Figure 5 in Cohen 2002). This suggests that different suites of antigens are selecting for differences in frequency in MH alleles resident in particular populations so that those associated alleles rise in frequency. Presumably, the effect is seen as a focus on particular binding pockets since those are codons involved in binding antigens common to that particular population.

Patterns of variation and response in other Fundulus populations

Because the *F. heteroclitus* populations from NBH have been studied comprehensively and from a multi-disciplinary perspective (reviewed in Nacci, Gleason and others 2002), they constitute an important and unique model. In addition, a number of other *F. heteroclitus* populations are being characterized that demonstrate a range of exposures and tolerances to PCBs (Nacci, Gleason and others 2002). This broader population survey suggests that PCB tolerance has evolved independently in several *F. heteroclitus* populations. For example, a population of *F. heteroclitus* residing in Bridgeport, Connecticut, a highly contaminated urban harbor, also shows PCB tolerance (D Nacci personal communication). These populations provide an unprecedented opportunity to test more generally the relationship between chemicals and pathogen exposures, immunogenetic change at the MH and realized immunological competence. Examination of MH II in the Bridgeport, CT population and other nearby less contaminated populations also shows population-specific patterns, in this case focused on binding pocket 4 (Figure 1).

The radar plot in Figure 1 shows the results of a comparison of amino acid substitutions at PBR sites located close together in the primary sequence and found in pocket 4, one of the 5 peptide-binding pockets of MHC class II. This type of analysis, described previously, shows population differences in frequencies of motifs at a single pocket rather than among pockets as in the comparison of NBH populations. On the 3-dimensional MHC class II molecules, sites 70, 71, and 78 are found clustered together in pocket 4 (Stern and others 1994). In Figure 1, in order to correct for uneven allelic sample sizes, frequencies of amino acid motifs in pocket 4 are represented as the number of alleles found in each population sample that contain the motif divided by the total number of alleles sampled. The amino acid motif EGT occurs in both of the reference populations at over 4 times the frequency found in the

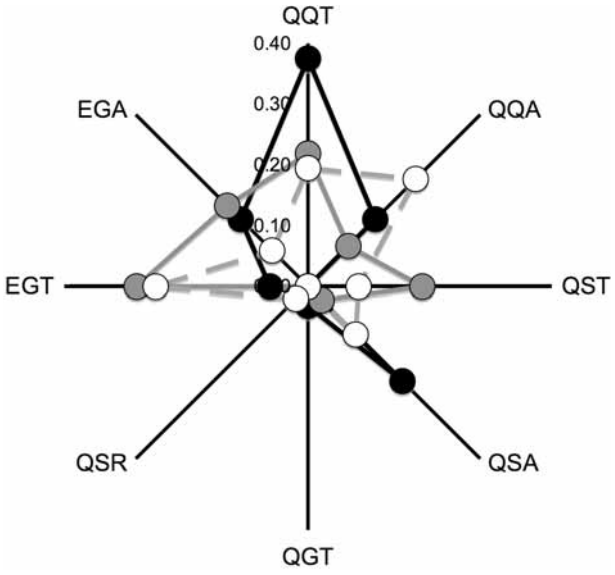

Fig. 1 Variation in amino acid substitution at PBR sites, pocket 4. MH IIDB peptide binding pocket-4 variation across killifish populations. The 3-letter combinations of amino acid motifs (for example, QQT and EGA) are comprised of sites 70, 71, and 78 of the mature, MHC class II protein (Stern and others 1994). Motif frequencies in the PCB-contaminated population, Bridgeport, Connecticut (number of alleles sampled = 32), are black circles. Motif frequencies in the reference populations Westport, Connecticut ($n = 32$) and Flax Pond, New York ($n = 36$) are light grey and white circles, respectively. All frequencies were divided by the total number of alleles to correct for uneven sample sizes.

contaminated population at Bridgeport. The amino acid motif QQT is also found at different frequencies in contaminated and reference populations, with an almost doubled frequency in Bridgeport compared to either reference population. Frequency differences of pocket-4 amino acid motifs between populations are inferred to represent the results of different selective pressures in the different locations, related to the extraordinary contaminant load at Bridgeport.

The likelihood that amino acid substitutions in pocket 4 represent a fingerprint of peptide-binding function is supported by previous MHC class II studies that have focused on variation in this area. Human and primate MHC class II studies have shown that variation in pocket-4 amino acids may alter peptide-binding function (Stern and others 1994; Diab and others 1999; Nino-Vasquez and others 2000), which in turn affects the resistance to pathogens. In the wild Malagasy mouse lemur, MHC class II alleles found to be associated with particular parasite loads also had unique substitutions at pocket-4 amino acid sites 70 and 71 (Schad and others 2005), suggesting a relationship between amino acid substitution within pocket 4, recognition of pathogens, and resistance to them.

Assessing immune function in fish population

Challenge experiments, the most direct tool for measuring the general competency of the immune system, have been successfully used to determine associations between MHC alleles and disease resistance and susceptibility to pathogens (reviewed by Piertney and Oliver 2006). Challenge experiments are additionally a sensible choice for many wild populations in view of the lack of immunological tools for directly evaluating MHC function and adaptive immunity (for example, Secombes and others 2005 for fish). We are currently conducting laboratory challenges (for example, using marine fish pathogenic bacterium such as *Vibrio harveyi*, Gauger and Gomez-Chiarri 2002) to evaluate immunological responsiveness among populations of *F. heteroclitus* indigenous to NBH and from less contaminated sites. Standardized bacterial challenges are being used to compare wild *F. heteroclitus* populations varying in factors potentially affecting immune responses, that is, MH class II loci, tissue concentrations of pollutants, and parasite type and loading. Experiments using field-collected and laboratory-bred fish are being conducted to separate genetic effects from environmental ones. Fish from these challenge experiments may be subsequently genotyped for MH class II loci to compare survival rates after bacterial challenges with particular functional motifs related to the structural antigen-binding model. Conversely, targeted challenge experiments may also be carried out in which fish with known genotypes of functional interest related to particular motifs in the structural model are tested, possibly in family designs where linkages may be carefully evaluated. These types of targeted challenge experiments may be particularly important in high-diversity genetic systems in which MH diversity seriously erodes statistical power in a more random, *post hoc* genotyping design.

The overall goal of this research is to test links between immunogenetic change and realized immunogenetic competence. While the acute laboratory challenges that we are performing have provided crucial information showing that long-term exposure to pollutants does not necessarily impair ability of fish to survive acute bacterial infection, it will be interesting to obtain further assessments as to whether they are realistic indicators of the outcome more typical of natural pathogenic exposures. For more direct assessment of functional implications of MHC differences among *F. heteroclitus* populations, methods for the experimental infection of fish by

cohabitation, a more natural route of infection, are anticipated. In addition, initial studies have been limited to bacterial pathogens. However, since MHC II molecules are associated mechanistically with antigenic responses to bacteria and parasites (Klein 1986), a more useful assessment may involve a suite of more typical pathogens/parasites affecting *F. heteroclitus* at these sites. For example, experimental infections of laboratory-bred fish from clean populations and those contaminated by parasitic pathogens, but more frequently observed in fish from NBH, will allow evaluation of the relative role of MH genetics, ecological factors, and pollutant level in the differences in parasitic loads observed among populations of *F. heteroclitus*.

The use of different methods of experimental infection can also provide some useful insights into the effects of long-term exposure to heavy contaminant loads on both the innate and the adaptive immune systems in these fish. Innate immunity plays a critical role during the first stages of infection and in the successful clearance of acute bacterial infections (such as the experimental infections of *F. heteroclitus* with *V. harveyi*) (Ellis 2001). Interestingly, strong activity of the innate immune system, as indicated by the respiratory-burst response, has been observed in 3-spined sticklebacks with suboptimal MHC diversity (Kurtz and others 2004). These results point to strong links between innate and adaptive immunity. There are a variety of innate immune parameters for which assays have been developed in fish, including respiratory burst, antimicrobial peptide response, lysozyme activity, and many more (Ellis 2001). Tools to measure the adaptive immune response of fish include antibody titer and affinity assays, cytokine levels, as well as T-cell proliferation assays (Secombes and others 2005). Recently, a method for measuring the non-specific proliferative response of T-cells to the plant lectin and mitogen phytohemagluttinin (PHA) has been successfully used to evaluate the immunosuppressive effect of certain pollutants on a small fish, *Betta splendens* (Ardia and Clotfelter 2006). There is a lack, however, of tools to measure specific T-cell responses of fish, since markers for the isolation of specific populations of T-cells have become available only recently (Secombes and others 2005). The study of the functional consequences of differences in MH class II in fish from sites with different contaminant pressure might ideally include individual assessments of parameters from both the innate and the adaptive immune systems. Characterization of the genetics and genomic structure of the MH in *Fundulus,* the patterns of expression of MH genes in fish immune cells in response to a variety of immune stimuli, as well as evaluation of the long-term and short-term effects of pollutants on MH expression, will also greatly benefit the study of the mechanisms of MHC selection in natural populations and the assessment of the functional consequences of such processes.

Conclusions

Studies of MHC variation in wild populations are appearing at a dizzying and exciting rate and provide an avenue for studying the mechanisms that create and maintain MHC diversity, as well as the functional consequences of that diversity. A combination of new tools and increased shared knowledge between evolutionary ecologists and immunologists has suddenly opened doors in the burgeoning field of ecological immunology and immunogenetics. To highlight just a few recent discoveries, an additional mode of selection that could promote high levels of MH polymorphism based on variable levels of gene duplication between individuals, "allele counting", was proposed for stickleback fish by Reusch and others (2001). Climatic cycling, tied to variation in MHC, parasite prevalence, and viability of offspring, was carefully explicated with an impressive 10-year database (Paterson and others 1998; Coltman and others 1999). Additional examples of MHC alleles conferring either susceptibility or resistance to particular pathogens have been delineated in natural systems, including genetically diverse species such as the hairy-footed gerbil (Harf and Sommer 2005). Some of the advances that made these insightful studies possible include an increasing ease of gathering MHC data, whether by various types of high-resolution gradient gels or by increasing ease of higher throughput cloning and sequencing. Beyond data collection, some critical advances in understanding MHC architecture have been made, in some cases facilitated by new large-scale genomic resources in increasing numbers of alternative taxa. These various advances in the understanding of MHC come from diverse species and wild populations across the vertebrate spectrum. The comparative method is a robust and informative approach in MHC ecological genetics now that some technical hurdles, such as finding highly variable antigen-binding regions in novel genomes, are being solved.

Novel, and often highly diverse, genetic systems present analytical challenges and provide pressing issues for MHC studies. In this review, we have attempted to highlight pressing issues in applying functional analysis to MHC studies both at the molecular level, making more detailed use of the crystal model for antigen binding, and at the statistical

level, taking advantage of the latest techniques in coalescent, Bayesian, and phylogenetic analysis where appropriate to MHC data. We hope that the intriguing aspects of MHC ecology and evolution in wild populations will continue to attract the interest of those developing structural modeling, bioinformatics, population modeling, and statistical techniques for treating highly variable data from population genetics.

Other exciting avenues in MHC research include the explicit tying of the functional MHC antigen-binding model to population-level analysis of susceptibility and tolerance, and the use of an integrated approach to immune function in wild systems, considering both innate and acquired immunity. The goal is to tie together diverse phenotypic measures of immune system function, as well as ecological studies on parasite prevalance and host-resistance measures, to more immediate and functional assays of cellular and humoral immune responses. Finally, the ability to use the MHC to study local selection in wild populations has opened an important avenue into the assessment of human impact in diverse environments. Classically, MHC has been studied in other vertebrate models, generally inbred strains, to consider issues in human health from an organismal and mechanistic viewpoint. MHC studies in wild populations offer the complimentary opportunity to study vertebrate health in an environmental context. *Fundulus heteroclitus*, an estuarine killifish tolerant of a wide range of natural and human-induced environmental stresses, offers an outstanding opportunity to learn about immunological genetics in the wild, in replicated habitats, and in laboratory and field experiments. We have begun to lay out a biocomplexity approach to immunogenetic health in this species, taking advantage of its abundance and its tolerance of varying estuarine quality. Using the MHC functional antigen-binding model, we are able to compare local population signatures of immunogenetic selection. We aim to combine these approaches with additional studies of parasite communities, host resistance, and other immunological responses for a fuller picture of both the species' health and the estuarine communities' health. These approaches, from the population genetic and biochemical/biophysical to the physiological and immunological, are poised to provide a rich tapestry of understanding of the functional mechanisms and consequences of variation in the MHC.

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