

*Review*

# How pathogens drive genetic diversity: MHC, mechanisms and misunderstandings

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Major histocompatibility complex (MHC) genes have been put forward as a model for studying how genetic diversity is maintained in wild populations. Pathogen-mediated selection (PMS) is believed to generate the extraordinary levels of MHC diversity observed. However, establishing the relative importance of the three proposed mechanisms of PMS (heterozygote advantage, rare-allele advantage and fluctuating selection) has proved extremely difficult. Studies have attempted to differentiate between mechanisms of PMS using two approaches: (i) comparing MHC diversity with that expected under neutrality and (ii) relating MHC diversity to pathogen regime. Here, we show that in many cases the same predictions arise from the different mechanisms under these approaches, and that most studies that have inferred one mechanism of selection have not fully considered the alternative explanations. We argue that, while it may be possible to demonstrate that particular mechanisms of PMS are occurring, resolving their relative importance within a system is probably impossible. A more realistic target is to continue to demonstrate when and where the different mechanisms of PMS occur, with the aim of determining their relative importance across systems. We put forward what we believe to be the most promising approaches that will allow us to progress towards achieving this.

**Keywords:** parasites; balancing selection; major histocompatibility complex; heterozygote advantage; rare-allele advantage; fluctuating selection

## 1. INTRODUCTION

Explaining how genetic variation is maintained in wild populations has long been a central question in evolutionary biology. Since previously unpredicted numbers of alleles were detected in populations of humans and *Drosophila* (Harris 1966; Lewontin & Hubby 1966), biologists have debated the relative roles of balancing selection and neutral processes in maintaining the diversity observed in wild populations (reviewed in Nei 2005). This debate has now matured, and the parsimonious appeal of neutral theory has led to it being accepted as the null hypothesis against which selection can be tested (Kreitman 1996). A number of genes believed to be subject to balancing selection (a bracket term encompassing a number of forms of selection that act to maintain multiple alleles within a population) have now been identified (Ford 2002). However, in the majority of cases, the exact causes and mechanisms behind the selection remain unclear. A major reason for this is that while DNA data have been relatively easy to collect, it has proved more difficult to identify gene function and more difficult still to show how variation in function is influenced by selection (Ford 2002). In wild-living organisms, finding suitable candidate genes for studying balancing selection is an especially difficult task, as the genetic basis of traits of interest is usually poorly understood.

Genes of the vertebrate major histocompatibility complex (MHC) arguably provide the most promising

opportunity for studying how balancing selection operates to maintain genetic variation in populations. The extensive population-level allelic richness (hereafter referred to as ‘diversity’) observed at these genes, alongside their central role in the vertebrate immune system, makes them ideal candidates for studying selection (Hedrick 1994; Meyer & Thomson 2001). The structure and function of MHC genes is now well understood in a range of organisms (e.g. Bjorkman *et al.* 1987; Sato *et al.* 1998; Kaufman *et al.* 1999; Hess & Edwards 2002), allowing testable hypotheses to be formed concerning the nature of selection operating on these genes.

It has long been suggested that pathogen-mediated selection (PMS) is the driving force maintaining diversity at MHC loci (Doherty & Zinkernagel 1975; Jeffery & Bangham 2000; Bernatchez & Landry 2003). Gene conversion and recombination, sexual selection and maternal–foetal interactions may also play a role, though these factors are outside the scope of this paper (for extensive reviews, see Edwards & Hedrick 1998; Martinsohn *et al.* 1999; Penn & Potts 1999). Three main hypotheses of PMS have been proposed: heterozygote advantage (Doherty & Zinkernagel 1975), rare-allele advantage (Slade & McCallum 1992) and fluctuating selection (Hill 1991). A strong theoretical framework has been established supporting the idea that any of the three mechanisms, or any combination of the three, could drive MHC diversity (Hughes & Nei 1988; Takahata & Nei 1990; Apanius *et al.* 1997). However, it has proved much more difficult to identify and

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differentiate between them empirically in wild populations, and so the key aim of determining the relative importance of the different mechanisms has remained elusive (Bernatchez & Landry 2003; Piertney & Oliver 2005). Early MHC studies focused on detecting selection operating over macro-evolutionary time scales, using methods based on coalescence theory or differences in synonymous and non-synonymous substitution rates (Hughes & Nei 1988; Takahata & Nei 1990). These methods proved useful for detecting the historical presence of selection on genes, but tell us little about the timing or nature of selection (Garrigan & Hedrick 2003). More recently, there has been increasing interest in examining selection at the MHC either within a single generation or over ecological time scales. Doing so allows for the detection of recent selection events and has led people to attempt to differentiate between mechanisms of PMS in non-model organisms. Indeed, over the last few years, the number of studies examining contemporary selection at the MHC has been overwhelming. Yet how much these studies have delivered, whether they are being conducted appropriately and how our understanding of MHC evolution is developing as a result have yet to be questioned.

A number of excellent reviews on MHC evolution have been published (Edwards & Hedrick 1998; Meyer & Thomson 2001; Bernatchez & Landry 2003; Garrigan & Hedrick 2003; Piertney & Oliver 2005; Sommer 2005); however, none have explicitly addressed the question of *if* and *how* we can differentiate between mechanisms of PMS. Our aim here is to assess the extent to which empirical studies have been able to do this. First, after a brief introduction to the MHC, we describe in detail the three mechanisms of PMS. This is important as some confusion appears to exist in the literature with regard to these hypotheses. Second, we provide a critique of the approaches used to detect selection in contemporary populations. We then describe how the outcomes of each approach relate to the different mechanisms of PMS and review the current MHC literature in this context. Finally, we highlight the major problems with current approaches to MHC research and discuss whether we are ever likely to be able to determine the relative roles of different mechanisms of PMS in maintaining MHC diversity in the wild.

## 2. MHC STRUCTURE AND FUNCTION

MHC genes code for molecules that bind to both self-peptide and non-self-peptide antigens, and present them to T-cells, thereby triggering a cascade of immune responses (Klein 1986; Potts & Wakeland 1990; Edwards & Hedrick 1998; Meyer & Thomson 2001). MHC molecules only bind peptides with anchor residues (amino acids at specific positions) that fit exactly to their binding pocket. As amino acids at other positions do not affect binding, each MHC molecule may bind to a range of different peptides.

MHC genes exist in a multigene family with two main subfamilies (class I and class II). Owing to differences in the pathways involved in antigen presentation, class I genes are associated primarily with intracellular pathogens and class II genes with extracellular pathogens (Jensen 2007). Another feature of the MHC is that different taxa exhibit different rates of gene conversion and

recombination in this region. This has led to many MHC genes being found in multiple, tightly linked copies, making it extremely difficult to identify and isolate independent loci, with the consequence that studies are often only able to amplify multiple loci (e.g. Binz *et al.* 2001; Richardson & Westerdahl 2003; Babik *et al.* 2009).

## 3. MECHANISMS OF SELECTION AT MHC LOCI

The *heterozygote advantage* hypothesis proposes that individuals heterozygous at MHC loci are able to respond to a greater range of pathogen peptides than homozygotes and, consequently, benefit from increased resistance to pathogens. Heterozygotes are, therefore, more likely to have higher relative fitness and, as a result, on average more MHC alleles will persist in the population (Doherty & Zinkernagel 1975; Hughes & Nei 1988).

Heterozygote advantage can be, and has been, a confusing concept for a number of reasons. First, heterozygote advantage can occur through both dominant and overdominant selection. If pathogen resistance is dominant, the heterozygous genotype exhibits the same level of fitness as the fittest homozygote (but not higher) and so achieves higher levels of fitness than the *average* for all homozygotes. If it is overdominant then the combined, synergistic effect of two alleles at a locus will result in the MHC heterozygote being fitter than the fittest homozygous genotype (Hughes & Nei 1988). This distinction is important because dominance alone cannot maintain diversity within individual populations, whereas overdominance can (Takahata & Nei 1990; McClelland *et al.* 2003), although both may operate to maintain diversity across metapopulations (see fluctuating selection hypothesis below). Second, because infection with a given pathogen will result in more than one non-self-peptide being present in the host, the heterozygote advantage gained by being able to detect a wider range of peptides can operate in response to both single (Kurtz *et al.* 2004) and multiple (McClelland *et al.* 2003; Wegner *et al.* 2003b) pathogens. In a population subject to heterozygote advantage, one may therefore expect to observe associations between MHC heterozygosity and both pathogen load and diversity. Nonetheless, heterozygote advantage may be best understood when considered in the context of multiple pathogens, as MHC alleles conferring resistance to one pathogen can increase susceptibility to another (Penn & Potts 1999). Finally, it has been argued that, at the individual level, it is not maximized but rather optimized heterozygosity that provides maximum fitness benefits; having too high MHC diversity may have diminishing returns on T-cell diversity, owing to the deletion of T-cells that react with self-peptide–MHC combinations during development (Nowak *et al.* 1992). This ‘optimal’ theory has received considerable empirical support (Wegner *et al.* 2003a; Kalbe *et al.* 2009). Therefore, studies examining maintenance of population-level MHC diversity should consider the immunological constraints on intra-individual diversity in order to fully understand the processes underlying selection at these genes.

The *rare-allele advantage* (also called negative frequency-dependence) hypothesis proposes that there is strong selection on pathogens to overcome the

resistance of the most common host MHC alleles. Therefore, new alleles that arise within the population are likely to offer greater protection to pathogens than common alleles, and so have a selective advantage (Takahata & Nei 1990). Old, rare alleles may also be selected for; an allele may decrease in frequency within a population owing to pathogens evolving resistance, but once the allele becomes rare, the pathogen adaptation may decrease or disappear, causing the selective advantage of the allele to increase again. The result of this process is a cyclical, coevolutionary arms race in which pathogens and MHC alleles fluctuate in frequency, thus maintaining diversity via a dynamic process (Slade & McCallum 1992).

Finally, the *fluctuating selection* hypothesis proposes that spatial and temporal heterogeneity in the type and abundance of pathogens may maintain diversity at the MHC (Hill 1991). If the pathogen regime faced by an organism fluctuates spatio-temporally, the intensity of directional selection at MHC genes will also fluctuate. This will lead to different subsets of MHC alleles being selected for at different points in space and/or time, thus maintaining genetic diversity across subpopulations. Key to this model is (i) that selection is directional, rather than cyclical and (ii) that pathogen fluctuations are determined externally—by the biotic or abiotic environment or chance dispersal and extinction events—rather than by coevolution of host and pathogen. Hedrick (2002) showed that, theoretically, diversity at the MHC could be maintained via fluctuating selection, even in the absence of heterozygote and rare-allele advantage.

Defining the mechanisms of PMS is, however, the easy part. Determining their relative roles in maintaining MHC diversity is another matter as they are by no means mutually exclusive and may operate in concert with other selective and neutral forces (Apanius *et al.* 1997). Moreover, the mechanisms may interact with one another. For example, there is an inherent frequency-dependent component in the heterozygote advantage model, and *vice versa*; because individuals are unlikely to inherit two copies of a rare allele, such alleles will occur disproportionately in heterozygous individuals. The intensity of selection on an allele under heterozygote or rare-allele advantage may also vary in space and time, owing to fluctuations in pathogens.

#### 4. EMPIRICAL EVIDENCE FOR THE NATURE OF SELECTION ON MHC GENES

Selection upon genes in contemporary populations is expected to produce detectable effects on the distribution of alleles within those populations (Meyer & Thomson 2001; Hedrick 2002). Therefore, by comparing patterns of variation at MHC genes with those expected under neutrality, one can make inferences about the nature of selection. Contemporary selection can also be revealed by examining pathogen load in relation to MHC characteristics. These approaches have been used not only to detect the presence of PMS, but also to try to differentiate between the models. However, it is often unclear exactly how the different results that arise from these approaches relate to different mechanisms of PMS, and so conclusions are often ambiguous. In an attempt to clarify predictions, in table 1 we summarize the ways in which

selection at MHC loci can be detected in contemporary populations, alongside the possible observations that arise from each of these approaches. We also state the mechanisms of PMS that we predict to be compatible with each observation. In what follows, these predictions are discussed in light of the empirical studies that have attempted to differentiate between mechanisms of PMS.

##### (a) *Proportions of genotypes within populations*

Many studies have attempted to detect selection at MHC genes over a single generation by comparing the distribution of MHC genotypes with a theoretical neutral distribution. Balancing selection is expected to result in a surplus of heterozygotes, and this was originally interpreted as evidence of heterozygote advantage (Doherty & Zinkernagel 1975). However, a simple excess of heterozygotes at MHC loci may also be compatible with rare-allele advantage, as heterozygotes may be selected for because they carry rare, resistant alleles, rather than because they are heterozygous *per se* (Penn 2002; table 1). Nonetheless, a number of studies have used this method, though few have detected a heterozygote excess (Paterson *et al.* 1998; Gutierrez-Espeleta *et al.* 2001; Penn *et al.* 2002; Seddon & Ellegren 2004; Oliver *et al.* 2009a). This may be because in many cases selection is not strong enough to be detected within a single generation.

An alternative approach is to examine the frequency of alleles in populations, which can provide a 'snapshot' of ongoing evolutionary processes. Alleles are unlikely to occur at equal frequencies. Instead, under neutrality, we expect a few common alleles, with the rest occurring at a relatively low frequency. By calculating a theoretical neutral distribution and contrasting it with empirical observations, it is therefore possible to detect selection (Ewens–Watterson test; Ewens 1972; Watterson 1978). However, because the Ewens–Watterson test is based on patterns of heterozygosity, it is still not possible to confidently differentiate between heterozygote advantage and rare-allele advantage using this method alone (table 1). Studies that have used the Ewens–Watterson test to assess selection at the MHC have found that the presence and/or strength of selection varies across subpopulations, with selection being detected in 15 to 50 per cent of subpopulations (Garrigan & Hedrick 2003). Such findings could represent fluctuating selection, spatially heterogeneous patterns of heterozygote advantage or snapshots of ongoing rare-allele advantage at varying points in evolutionary time (table 1). Furthermore, the Ewens–Watterson test assumes constant population size, so departures from neutrality calculated using this method may arise owing to historical demographic processes (Nei 1987). The varying results from the studies that have used this test may, therefore, be due to differential demographic histories across populations and not because of fluctuations in selective forces. One way to circumvent the problems associated with a theoretically derived neutral allelic distribution has been to assess this distribution directly using neutral markers (Boyce *et al.* 1997). While demographic processes affect all loci, selection targets specific genes; therefore, by contrasting patterns of variation at MHC and neutral loci, the effects of demography can be controlled for.

Table 1. Predictions arising from different models of pathogen-mediated selection (PMS). Note that selection is assumed here—the effects of drift must be controlled for by measuring neutral variation empirically (see text).

	possible observations	possible PMS explanations
proportions of genotypes within populations	an excess of heterozygotes	dominance, overdominance, rare-allele advantage (as rare alleles will normally exist in heterozygotes)
	an excess of homozygotes	underdominance
	an excess of heterozygotes in a subset of populations studied no deviation from neutral expectations	fluctuating selection, fluctuations in any mechanism neutrality
distribution of allelic frequencies within populations	more even than expected under neutrality	dominance, overdominance, rare-allele advantage
	less even than expected under neutrality	directional selection, fluctuating selection if variable across multiple populations
	more even than expected under neutrality in a subset of populations studied	fluctuating selection, fluctuating dominance, fluctuating overdominance, rare-allele advantage
	no deviation from neutral expectations	neutrality
levels of population structure relative to neutral loci across populations	higher population structure at MHC compared with neutral loci	fluctuating selection, rare-allele advantage maintaining new alleles
	lower population structure at MHC compared with neutral loci	dominance, overdominance, rare-allele advantage selecting old alleles
	equal population structure at MHC compared with neutral loci	neutrality
associations with pathogen regime	associations between individual pathogen load and MHC alleles	rare-allele advantage, fluctuating selection
	association between individual pathogen load and MHC heterozygosity	heterozygote advantage (to a single pathogen); overdominance if heterozygote associated with lower pathogen load than both homozygotes
	association between individual pathogen load and number of MHC alleles (across multiple duplicated loci)	heterozygote advantage, rare-allele advantage, fluctuating selection
	associations between pathogen diversity and individual MHC alleles	none
	association between pathogen diversity and MHC heterozygosity	heterozygote advantage (to multiple pathogens); overdominance if heterozygote associated with fewer pathogens than both homozygotes
	associations between pathogen diversity and number of MHC alleles (across multiple duplicated loci)	heterozygote advantage, rare-allele advantage, fluctuating selection
	no association between MHC structure and pathogen regime	neutrality

Incorporating neutral variation into tests of selection has highlighted that migration and drift may sometimes be more important than selection in shaping MHC diversity over micro-evolutionary time scales. Indeed, many studies have reported that neither MHC nor neutral variation differ from random expectations (Bernatchez & Landry 2003). However, in any system where multiple forces act on allelic distributions, there is the possibility that they may cancel each other out to produce patterns that do not deviate from neutral expectations. On the other hand, a few studies have reported selection at MHC loci after controlling for demographic processes, though only in subsets of populations. Studies on salmonids have reported elevated heterozygosity at MHC compared with neutral loci in 16 to 43 per cent of populations (Landry & Bernatchez 2001; Miller *et al.* 2001; Aguilar & Garza 2006). Recently, Oliver *et al.* (2009a) reported elevated heterozygosity in metapopulations of water voles (*Arvicola terrestris*), but only in 3 out of 10 metapopulation-years. Authors tend to attribute

these findings to differential selection pressures arising from spatio-temporal variation in pathogen abundance, though that it is due to its dynamic nature, rare-allele advantage cannot be ruled out. Westerdahl *et al.* (2004) adopted a temporal approach, comparing variation in nine successive cohorts of great reed warblers (*Acrocephalus arundinaceus*). Overall, variation in the frequency of MHC alleles was significantly greater than for microsatellite alleles. Moreover, the frequency of two MHC alleles, but no microsatellite alleles, varied more between cohorts than expected, suggesting that selection favours different MHC alleles in different years—findings consistent with both the rare-allele advantage and fluctuating selection hypotheses. Hess *et al.* (2007) also observed temporal shifts in MHC allelic frequencies in populations of house finches (*Carpodacus mexicanus*), though their results were less pronounced.

Studies on bottlenecked populations have found higher levels of MHC compared with neutral diversity, indicating that balancing selection can act to counter

demographic processes. For example, the San Nicolas Island fox (*Urocyon littoralis dickeyi*) is reportedly the most genetically monomorphic sexually reproducing animal population, having gone through a bottleneck of less than 10 individuals (Aguilar *et al.* 2004). Despite this, high levels of diversity were observed at three MHC-linked microsatellites and one class II gene (Aguilar *et al.* 2004). Similar, though less extreme, elevated MHC diversity relative to neutral loci has been observed in bottlenecked populations of several other species (e.g. Richardson & Westerdahl 2003; Jarvi *et al.* 2004; Hansson & Richardson 2005; van Oosterhout *et al.* 2006).

### (b) Patterns of population structure

Selection can also be detected by contrasting population structure at MHC and neutral loci across multiple populations (table 1). In populations subject to heterozygote advantage, within-population MHC diversity is predicted to be high compared with total diversity, resulting in a lower population structure at MHC than at neutral loci (Schierup *et al.* 2000). Conversely, in populations subject to fluctuating selection, different subsets of MHC alleles will arise, and higher population structure will be observed at MHC relative to neutral loci (Charlesworth *et al.* 1997). The rare-allele advantage model adds confusion to this picture, as we know little about the nature of the rare alleles themselves. Obviously, only rare alleles that currently confer resistance to pathogens are selected. However, whether these alleles are predominantly old (i.e. ones that have previously been common within the population) or newly arisen will have different consequences for patterns of population structure. If they are old, population structure will be lower at MHC than neutral loci (Schierup *et al.* 2000). If newly arisen alleles are selected for, then different subsets of alleles will arise in different populations, and consequently structure will be relatively higher at the MHC.

Studies comparing population structure at MHC and neutral loci have yielded mixed results. Many studies have found no difference between MHC and neutral population structure (e.g. Boyce *et al.* 1997; Gutierrez-Espeleta *et al.* 2001; Babik *et al.* 2008; Biedrzycka & Radwan 2008), suggesting that little or no selection is operating across populations, or that multiple selective forces are operating and masking any overall effects. On the other hand, differentiation at MHC relative to neutral loci across populations has been reported, for example, in various fish species (Landry & Bernatchez 2001; Miller *et al.* 2001; Aguilar & Garza 2006) and in great snipe (*Gallinago media*; Ekblom *et al.* 2007). In all these studies, the authors conclude that MHC diversity is maintained by different patterns of PMS across differing ecological environments. Alcaide *et al.* (2008) also reported elevated MHC differentiation across populations of lesser kestrel (*Falco naumanni*). In this case, as the kestrel populations inhabit similar habitats—with presumably similar pathogen communities—the authors conclude that this pattern has arisen from geographically varying coevolution, supporting the rare-allele advantage model of selection. However, none of these studies demonstrate explicitly that the pathogen fauna does, or does not, vary across populations. Such studies are badly needed; without them attempts at differentiating between

rare-allele advantage and fluctuating selection are speculative at best. Very few studies have detected lower levels of divergence at MHC than at neutral loci. van Oosterhout *et al.* (2006) found lower differentiation at MHC class II DAB genes than at microsatellite loci across two populations of Trinidadian guppies (*Poecilia reticulata*). These results suggest that MHC diversity may be maintained either by heterozygote advantage or by rare-allele advantage in which old, rare alleles are maintained. Two other studies have compared MHC and mitochondrial genes and found lower  $F_{ST}$  values at MHC loci (Sommer 2003; Mona *et al.* 2008); however, the use of mitochondrial genes as a neutral marker in this manner is questionable (William *et al.* 1995).

This brings us to a major problem intrinsic to studies that compare MHC and neutral variation; namely, which markers are most appropriate for assessing neutral diversity. Most studies have used microsatellites, presumably because they allow an efficient, low-cost assessment of neutral diversity. However, these studies are confounded by the fact that the manner and rate in which microsatellites and MHC sequences mutate are very different. Specific statistical tests are often required for the different markers, and the outcomes of tests that can be used with both markers may be affected by their differential mutation rates (Hedrick 2005; Brito & Edwards 2009). A logical solution to this problem would be to use nuclear sequence polymorphisms, which are likely to evolve in a fashion more similar to MHC genes (Brito & Edwards 2009), though this has rarely been done.

### (c) Associations with pathogens

Contemporary selection at MHC genes may also be detected by examining associations between the MHC and pathogen load of an organism (table 1). Associations between specific pathogens and particular MHC alleles suggest a role for either rare-allele advantage or fluctuating selection. Associations between MHC heterozygosity and pathogen abundance and/or richness suggest a role for heterozygote advantage, though other mechanisms cannot be ruled out from this observation alone. Studies of this kind should also measure neutral variation to control for potential confounding effects of demographic processes on MHC structure. Those that have done so, described below, constitute the most detailed examples of how PMS can act to maintain MHC diversity.

In house sparrows (*Passer domesticus*), Bonneaud *et al.* (2006) found that two different MHC alleles were associated with resistance to the same malarial strain in different populations. Both alleles exist in both populations, indicating that local adaptation to malarial infection occurs. This study provides the strongest available evidence supporting rare-allele advantage, though a temporal component to the association between alleles and the pathogen would need to be incorporated to rule out other mechanisms completely. In great reed warblers, Westerdahl *et al.* (2005) found that the number of MHC class I alleles in an individual—a measure suggested to reflect heterozygosity across multiple loci—was associated with the presence of a particular malarial lineage, whereas heterozygosity measures derived from microsatellite data did not show this relationship. A single MHC allele was also associated with presence of the malarial lineage.

While these findings indicate that PMS is important in the great reed warbler, they are compatible with all three mechanisms of selection. Other studies have screened for multiple pathogens. In three-spined sticklebacks (*Gasterosteus aculeatus*), Wegner *et al.* (2003b) detected a positive correlation between the number of alleles across MHC loci (but not at microsatellites) within a population and population-level pathogen diversity, suggesting a role for heterozygote advantage, though other mechanisms could not be ruled out. Oliver *et al.* (2009b) contrasted MHC class II variation and pathogen regime in a population of water voles in which only two alleles, and thus three genotypes, are found. The heterozygous MHC genotype was associated with lower numbers, and fewer types, of ectoparasites. Importantly, the heterozygotes were more resistant than *both* homozygotes, indicating heterozygote advantage through overdominance. An association between infection with a specific parasite and an MHC allele was also detected, suggesting that rare-allele advantage and/or fluctuating selection may also operate. Finally, in populations of montane water voles (*Arvicola scherman*), Tollenaere *et al.* (2008) found an association between specific MHC alleles and pathogens, but no relationship between pathogen diversity and MHC diversity. This may suggest a greater role for rare-allele advantage and/or fluctuating selection and a lesser role for heterozygote advantage in this system. Overall, these studies indicate that all three mechanisms of PMS may operate to maintain MHC diversity in wild populations, but they do not allow us to rule out alternative mechanisms nor determine their relative importance.

## 5. CAN WE DIFFERENTIATE BETWEEN MECHANISMS OF PMS IN WILD POPULATIONS?

The number of studies examining selection at the MHC over a micro-evolutionary time scale has increased rapidly over the last few years. This work has highlighted that while selection at MHC genes is almost always detected over macro-evolutionary time scales, in contemporary populations MHC diversity is shaped by a range of neutral and selective forces, any combination of which may be operating at particular points in space and time. The research has confirmed the role of pathogens in MHC evolution and highlighted that pathogens and MHC genes interact closely in a number of ways, and that these interactions vary spatio-temporally. Some progress has been made in studying the mechanisms of PMS and the evidence, though circumstantial, now suggests that all three mechanisms may operate in natural systems.

Collating studies that have attempted to differentiate between mechanisms of PMS have shown that in many situations, the three mechanisms can produce the same effects on MHC diversity. Indeed, as table 1 shows, in the majority of cases, there are multiple explanations for any observation in a given test, and no one approach yields observations that allow the mechanisms to be differentiated between. To complicate matters further, the mechanisms are likely to interact, and other evolutionary processes, such as sexual selection, are also likely to contribute towards shaping MHC diversity (reviewed in Penn & Potts 1999). For example, MHC-based mate choice may often serve as an 'amplifier' to PMS, helping

to achieve an optimal or maximal number of MHC alleles (Richardson *et al.* 2005; Jager *et al.* 2007; Eizaguirre *et al.* 2009). However, if PMS within a population is directional (and so reduces diversity) and mate choice operates to maintain diversity, the two may effectively cancel each other out (Apanius *et al.* 1997).

This leads us to ask: what can we resolve, and how can we best move forward? By contrasting MHC variation with pathogen load and/or survival, it should be possible to detect the presence of heterozygote advantage, though few studies have done this convincingly in wild populations (but see Oliver *et al.* 2009b). Doing so requires examining associations between pathogens and *both* genotypes (heterozygosity) and specific alleles. It also requires a sufficient range of pathogens and an appropriate MHC screening method (i.e. single-locus amplification). Therefore, though the lack of heterozygote advantage observed so far may be because this mechanism is relatively unimportant in maintaining MHC diversity, it may also be because appropriate study systems have proved difficult to find.

A more serious challenge lies in separating rare-allele advantage and fluctuating selection. None of the approaches listed in table 1 enables us to tease apart these mechanisms as their effects upon MHC allelic frequencies in populations and on associations between MHC and pathogen structure are likely to be the same. A number of studies infer the importance of one mechanism even though their results cannot rule out alternatives. In particular, studies claim evidence of fluctuating selection after finding different levels of balancing selection across populations, or higher levels of population structure at MHC than at neutral genes (Landry & Bernatchez 2001; Miller *et al.* 2001; Aguilar & Garza 2006; Ekblom *et al.* 2007; Alcaide *et al.* 2008; Oliver *et al.* 2009a). However, as we have explained (table 1), such patterns could be due to different intensities of heterozygote advantage, or specific forms of rare-allele advantage. Combining observations of allelic frequencies with the genealogy of MHC alleles may go somewhere towards disentangling these effects, as new, rare alleles are expected to be less divergent than older ones.

The best way to differentiate between rare-allele advantage and fluctuating selection would be to study MHC and neutral variation in relation to pathogen load over periods of evolutionary time in multiple replicate populations. Under rare-allele advantage, one would expect to see different alleles conferring resistance to the same pathogen in different populations, and for resistance to change with time, so that different alleles become associated with resistance. Under fluctuating selection, one would expect to observe external biotic and/or abiotic forces driving spatio-temporal variation in pathogen abundance, leading to distinct subsets of alleles being selected for in different populations and/or different time periods. Of course, such long-term, multiple-population studies are difficult and costly to carry out, and appropriate study systems are difficult to find.

As well as the theoretical problems with teasing apart mechanisms of PMS, there are technical issues that also need to be resolved if we are to progress towards a fuller understanding of MHC evolution. First, although much of the variation in findings from MHC studies may have arisen because MHC evolution is indeed sporadic, it is

highly likely that poor quality control in the studies themselves has also confused matters. In particular, an inability to assign alleles to individual loci means that it is often not possible to identify true genotypes, making it difficult to employ many of the analyses used to separate mechanisms of PMS. The only way to circumvent this problem will be to use single-locus systems that allow for identification of true genotypes (e.g. Worley *et al.* 2008; Oliver *et al.* 2009b). An equally serious problem is that it is often unknown whether the MHC loci being studied are actually expressed, or whether diversity at these loci is being fully characterized. Detailed molecular groundwork is required to ensure that the full complement of expressed MHC variation is accurately assessed. Real-time PCR can be used to assess patterns of MHC expression in non-model organisms, though few studies have done so thus far (but see Wegner *et al.* 2006). The use of next-generation sequencing for MHC screening (Babik *et al.* 2009) is likely to be a great help in terms of more accurately characterizing MHC diversity, and we expect to see an increase in both the efficiency and resolution of MHC genotyping in non-model organisms in the near future.

Even when all the variation at a particular MHC locus is screened, we are faced with the problem of what equates to a functionally important MHC allele. Clearly, two alleles that differ by multiple amino acids are going to be able to detect a broader spectrum of antigens than two that differ by a single substitution. Yet allelic divergence is rarely taken into account in MHC studies, and so it is questionable whether these studies have classified biologically meaningful alleles. One way to obviate this problem could be to group sequences into functionally important 'supertypes' (Doytchinova & Flower 2005; Naugler & Liwski 2008). Another may be to quantify levels of amino acid divergence and use this in analyses.

A final 'technical' issue to consider is that MHC study systems are usually confounded by the very thing that makes the MHC variation an attractive subject to study—namely complexity. Most organisms are faced with enormous numbers of pathogens and are, in turn, characterized by a highly complex MHC. Is it any wonder therefore that studies contrasting a single exon of an MHC locus with individual pathogens produce mixed results? Fully characterizing the MHC and pathogen load is unlikely to be possible in most study systems, and even if it were, statistical analysis may be intractable. One rewarding approach could be to focus on highly simplified study systems (e.g. Richardson & Westerdahl 2003; Oliver *et al.* 2009b), though whether results from such studies are applicable to more complex systems is questionable. The best study systems will probably be characterized by intermediate levels of pathogen diversity and simple, well-characterized MHC structures, thus avoiding oversimplification while retaining statistical tractability.

Experimental infection studies may also help alleviate some of the problems associated with differentiating between mechanisms of PMS. A number of experimental infection studies with MHC congenic mice have shown that heterozygote advantage, through both dominance and overdominance, can operate to combat pathogenic infection (reviewed in Penn 2002). Experiments that differentiate between rare-allele advantage and fluctuating

selection have yet to be designed, perhaps because doing so would require long-term, controlled selection lines, with more generations than would be realistically possible in a vertebrate (though see Conover & Van Voorhees 1990). Even so, if this problem can be circumvented, the advantage of being able to control for demographic processes and external fluctuations in pathogen load may be extremely useful in differentiating between these two mechanisms.

Given that in the majority of cases the three mechanisms of PMS produce similar final effects on MHC dynamics, and that they are likely to interact, is differentiating between them in wild populations actually important? We believe so: understanding how diversity is maintained in gene regions such as the MHC is fundamental to our understanding of natural selection and antagonistic coevolution. Experimental infections, though more controlled, are limited in the information they can provide about the dynamics of PMS. Moreover, balancing selection clearly operates in different ways across ecologically and evolutionarily differing environments. Only by assessing MHC diversity in the wild will we be able to further understand these patterns and processes. Furthermore, there are applied implications for understanding how PMS operates to maintain MHC diversity in the wild, such as in conservation. For example, different mechanisms of PMS are likely to produce different phenotypic and population-level effects in response to the introduction of novel pathogens—a phenomenon that is likely to increase with a warming global climate (Smith *et al.* 2009). Reduction and fragmentation of populations will change pathogen dynamics and are likely to affect the nature of selection acting upon MHC genes (van Oosterhout *et al.* 2006). Understanding how organisms are likely to respond evolutionarily to factors such as genetic bottlenecks requires knowledge of how PMS operates to maintain diversity. Such understanding may also help us determine how best to conserve diversity at the MHC—or, for that matter, at any immunologically important genes—in wild populations, or how to maximize diversity through selective breeding in captive populations.

## 6. CONCLUSIONS

Owing to the non-exclusivity of the mechanisms of PMS, alongside the likely interference of other selective and neutral forces, we do not believe that it is possible to convincingly elucidate the relative roles of mechanisms of PMS within specific wild populations. A more realistic approach is to attempt to demonstrate whether a particular mechanism of PMS is occurring within a specific system. Doing so will be extremely difficult, not least because appropriate long-term study systems are vital for this. Researchers of contemporary MHC evolution therefore need to carefully consider which mechanisms of PMS can be detected within their study system and which can be ruled out, and interpret their findings accordingly. Technical issues concerning the characterization of expressed MHC variation also need to be carefully considered, as they have not been in much of the MHC literature to date. Nonetheless, with enough appropriate studies, we may be able to determine how often different mechanisms of PMS occur, as well as the

different spatial and temporal scales at which they prevail. The accumulation of such knowledge across systems will allow us to evaluate the general importance of the different PMS mechanisms across vertebrates as a whole. Advancements in our understanding of what maintains MHC diversity will also feed into our general understanding of host–pathogen coevolution and the maintenance of genetic diversity.

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