Major histocompatibility genes, polymorphism and balancing selection

- the case of parasites and sticklebacks

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K. Mathias Wegner

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Referent: Dr. habil. Thorsten B. H. Reusch

Korreferent: Prof. Dr. Thomas Bosch

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To create is to recombine.

Francois Jacob (1977)

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Summary

The extreme polymorphism of genes of the vertebrate major histocompatibility complex is an evolutionary puzzle. Why do high numbers of alleles persist despite selective forces favouring the fittest variants? MHC molecules present peptides to T cells initiating antibody production and thereby trigger the adaptive immune response. Since a given MHC molecule can only present a limited number of different peptides, an immune response can only be initiated against a limited amount of pathogens per MHC allele (Doherty and Zinkernagel 1975). As early as 1966 without any knowledge of form and function, selective mechanisms were proposed (Clarke and Kirby 1966) and MHC polymorphism is thought to be a result of balancing selection. Balancing selection mainly operates by two mechanisms: Overdominance and negative frequency dependent selection.

In overdominant selection heterozygotes have higher fitness than both homozygotes – a likely scenario for MHC genes, because MHC heterozygotes will be able to present more pathogen derived peptides to T cell than homozygotes. However, on theoretical grounds maximal heterozygosity at all MHC genes is not beneficial, because with a higher number of MHC alleles more self-reactive T cell lines get eliminated (Nowak et al. 1992; Borghans et al. 2003).

Negative frequency dependendance is based on antagonistic co-evolution between hosts and parasites, also known as the Red Queen hypothesis (Van Valen 1973). Parasites should track common host genotypes giving rare genotypes a selective advantage.

Using three-spined sticklebacks and their natural parasite fauna as a model organisms, this thesis addresses some of the key mechanisms that may explain MHC polymorphism.

Is the distribution of MHC polymorphism linked to parasite infections in individuals as well as in populations? Diversity of stickleback MHC class IIB genes were measured as the number of alleles correlated with parasite diversity measured as Simpson's D in population across northern Germany (Wegner et al. 2003b, Chapter I). Within individuals those with an intermediate individual MHC diversity (i.e. the number of MHC class IIB alleles) were on average suffering least from parasite infection in the field (Wegner et al. 2003b, Chapter I). In controlled experiments (Wegner et al. 2003a, Chapter II) and semi-natural enclosures fish with intermediate MHC diversity survived

best (Chapter IV). This novel finding supports theoretical predictions of MHC optimality at intermediate levels (Nowak et al. 1992) much more directly than the previous indirect evidence stating that polyploids circumvent the negative consequences of increased MHC diversity by suppressing expression of surplus MHC genes (Du Pasquier et al. 1989; Dixon et al. 1996; vanErp et al. 1996; Kruiswijk et al. 2004). Also in sticklebacks, expression of MHC class IIB genes seems to be influenced by the individual MHC diversity. The higher the average number of MHC alleles within families the lower is their constitutive expression level of MHC class IIB genes (Chapter III), indicating that similar mechanisms might act to prevent disadvantages, when individual MHC diversity is too large.

The evidence for overdominant selection acting on MHC class IIB genes in sticklebacks is probably not enough to fully explain MHC polymorphism. Host-parasite co-evolution would further predict that the fitness of genotypes/alleles should fluctuate depending on its frequency. In three sticklebacks MHC class IIB alleles fluctuate stronger between consecutive generations than genetic markers not subjected to selection (i.e. microsatellites). These fluctuations could partly be linked to changes in parasite prevalence (Chapter V) and therefore provide support for antagonistic co-evolution between MHC genes and parasites.

Polymorphism of MHC genes in three-spined sticklebacks is tightly linked to infestation by parasites. Both mechanisms of balancing selection, overdominant and negative frequency dependent selection, mediated by parasite induced selection pressures synergistically maintain the polymorphism at MHC class II genes. Since female sticklebacks try to achieve the same optimal number of MHC class IIB alleles in their offspring by choosing a male, whose MHC alleles complements her own set of alleles (Reusch et al. 2001a; Aeschlimann et al. 2003), sticklebacks form a unique example for congruent aims of natural and sexual selection. Parasitism not only provides selection to maintain polymorphism in defense genes, but also to cause the evolution of highly sophisticated mate choice mechanisms.

Zusammenfassung

Der außerordentliche Polymorphismus des Hauptgewebekompatibilitätskomplexes (Major histocompatibility complex, MHC) in Wirbeltieren ist ein evolutionsbiologisches Rätsel. Warum bleibt eine hohe Anzahl von Allelen erhalten, obwohl natürliche Selektion die fittesten Varianten bevorzugt? MHC-Moleküle der Klassen I und II präsentieren T-Zellen Pathogenpeptide und initiieren damit die Produktion von Antikörpern und im Endeffekt der adaptiven Immunantwort. Da jedes MHC-Molekül nur eine begrenzte Anzahl von Peptiden binden kann, sind Pathogene in der Tat eine wahrscheinliche Ursache für die selektive Erhaltung von MHC-Polymorphismus (Doherty and Zinkernagel 1975). Bereits 1966, ohne jegliche Kenntnis über Form und Funktion von MHC-Molekülen, wurden selektive Mechanismen zur Erklärung des gefundenen Polymorphismus herangeführt (i.e. balancierende Selektion) (Clarke and Kirby 1966). Balancierende Selektion kann hauptsächlich durch zwei Mechanismen erklärt werden: Überdominante und negativ-frequenzabhängige Selektion.

Im Fall von überdominanter Selektion haben Heterozygote einen Fitnessvorteil gegenüber beiden Homozygoten – kein unwahrscheinliches Szenario für MHC-Gene, da MHC-Heterozygote in der Lage sind mehr verschiedene Peptide zu binden als Homozygote. Allerdings ist eine maximale Heterozygotät an allen MHC-Loci aufgrund von theoretischen Vorhersagen nachteilig, da mit steigender Anzahl von MHC-Allelen in Individuen auch mehr selbst-reaktive T-Zelllinien eliminiert werden (Nowak et al. 1992; Borghans et al. 2003).

Negativ-frequenzabhängige Selektion basiert auf antagonistischer Ko-evolution zwischen Wirt und Parasit – der sogenannten Red Queen-Hypothese (Van Valen 1973), die besagt, dass Parasiten bevorzugt häufige Wirtsgenotypen infizieren sollten. Daraus folgt ein selektiver Vorteil für seltene Genotypen, was zu einem unvermeidbaren Wettrüsten zwischen Wirt und Parasit führt.

Mit dem dreistachligen Stichling Gasterosteus aculeatus und dessen natürlicher Parasitenfauna als Modellorganismen versucht diese Arbeit einige der wichtigsten Fragen zum MHC-Polymorphismus zu beantworten.

Steht die Verteilung von MHC-Polymorphismus sowohl in Individuen als auch in Populationen in Verbindung mit der Wahrscheinlichkeit von Parasiteninfektionen? In natürlichen Populationen aus Norddeutschland wurde eine Abhängigkeit zwischen der Anzahl von MHC-Allelen und der Parasitendiversität gefunden (Wegner et al. 2003b, Chapter I). Individuell betrachtet waren die Individuen, die eine mittlere individuelle MHC-

Diversität hatten im Mittel am besten vor Parasitenbefall geschützt. Dies gilt fürs Freiland (Wegner et al. 2003b, Chapter I), für kontrollierte Experimente (Wegner et al. 2003a, Chapter II) und semi-natürlichen „Enclosures“ , in denen auch Individuen mit mittlerer MHC-Diversität die höchste Überlebenswahrscheinlichkeit zeigten (Kapitel IV). Diese Experimente bestätigen die theoretischen Vorhersagen von intermediärer MHC-Optimalität (Nowak et al. 1992) sehr viel direkter als die Unterdrückung der Transkription von MHC-Genen in polyploiden Arten (Du Pasquier et al. 1989; Dixon et al. 1996; vanErp et al. 1996; Kruiswijk et al. 2004). Allerdings ist auch in Stichlingen die Expression von MHC-Genen von der individuellen MHC-Diversität abhängig. Je höher die mittlere Anzahl an MHC-Allelen innerhalb einer Familie ist, desto weniger werden diese Gene expremiert (Kapitel III), was ein Anzeichen für ähnliche Kontrollmechanismen zur Vermeidung der negativen Konsequenzen darstellen könnte, die durch eine zu hohe individuelle MHC-Diversität entsehen.

Dass überdominante Selektion auf MHC Klasse IIB-Gene in Stichlingen wirkt ist wahrscheinlich nicht ausreichend, um den beobachteten Polymorphismus gänzlich zu erklären. Wirts-Parasiten Ko-evolution würde zudem postulieren, dass die Fitness von Genotypen zeitlich in Abhängigkeit ihrer Frequenz fluktuieren sollte. MHC-Allele von Stichlingen fluktuieren zeitlich stärker als genetische Marker, die nicht durch natürliche Selektion erfasst werden sollten (i.e. nicht-kodierende Mikrosatelliten). Diese Frequenzänderungen sind teilweise mit Frequenzänderungen in der Parasitenprävalenz gekoppelt (Kapitel V) und unterstützt damit die Vorhersagen antagonistischer Ko-evolution zwischen MHC-Genen und Parasiten.

Der Polymorphismus von MHC-Genen in dreistachligen Stichlingen hängt damit eng mit Infektion durch Parasiten zusammen. Beide Mechanismen balancierender Selektion tragen zur Erhaltung des Polymorphismus bei und werden jeweils von Parasiten gesteuert. Da Stichlingsweibchen zudem durch Partnerwahl versuchen die MHC-Diversität ihrer Nachkommen auf das intermediäre Optimum einzustellen (Reusch et al. 2001a; Aeschlimann et al. 2003), stellen Stichlinge eine einzigartiges Besispiel für Kongruenz von natürlicher und sexueller Selektion dar. Parasitismus würde damit nicht nur ausreichend starke Selektion zur Erhaltung des extremen Polymorphismud von MHC-Genen bewirken, sondern auch für die Evolution von hochkomplizierten Partnerwahlmechanismen.

Introduction

Parasitism – the fuel for rapid co-evolution

MacInnes (MacInnis 1976) defined parasitism as the interaction between two species in which the survival of one, the parasite, is dependent on at least one gene of the other, the host. This definition implies that the parasite exploits the resources of the host. This will inevitably involve costs for the host and thereby a selective pressure to avoid parasitism. The genotypes that are resistant towards infection will have a selective advantage and will in turn rise in frequency. In a mutual interaction this will exert selection on the parasite because susceptiple hosts will get increasingly rare and successful transmission is not guaranteed anymore. Selection will therefore favour new parasite variants, which can infect the common resistant host genotypes (Fig. 1). This arms-race situation describes the rapid dynamics of host-parasite co-evolution (Van Valen 1973).

It is generally accepted that host will never completely eradicate parasites in the arms race, because parasites usually have shorter generation times or larger effective population sizes, which raises their evolutionary potential above that of the host. To maintain genetic variation in order to generate enough resistant genotypes, other mechanisms than mutation are needed. Beneficial mutations are rare events and two independent mutations will seldom occur within the same germ line. Recombination by sexual reproduction is a mechanism, in which parental chromosomes get rearranged in the offspring, possibly combining beneficial or eliminating detrimental variants much faster than in asexuals (Hamilton et al. 1990; Maynard Smith 1998). However, sexual populations are not stable and can easily be invaded by asexuals. Therefore, sexual reproduction has a high

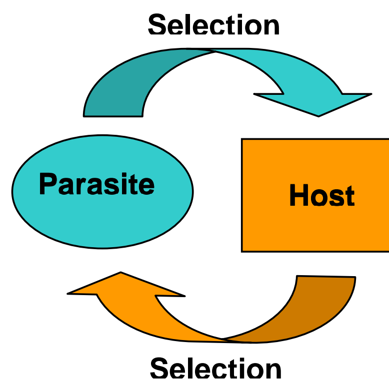


Figure 1: Schematic representation of host- parasite co-evolution. By using host resources the parasite imposes selective pressure on the host to to build up resistance. Resistant host genotypes impose in turn selection on the parasite to overcome host resistance – resulting in an arms-race situation.

cost – known as the two-fold cost of sex (Maynard Smith 1998). Why sexual reproduction is nonetheless the most common form of reproduction in the animal kingdom is an enigma of evolutionary biology. The rapid dynamics of host-parasite co-evolution are a promising hypothesis to explain the widespread occurrence of sex. Parts of these dynamics, also known as Red-Queen dynamics (Van Valen 1973), have been successfully demonstrated (Lively et al. 1990; Lively and Dybdahl 2000). The recurrent cycles of parasite and host genotypes predicted by Red-Queen theory and the role of sexual reproduction for resistance in host genotypes have so far been difficult to observe. Genes involved in host immunity are promising candidate genes to look for genotype-genotype interactions and dynamical changes of gene frequencies, because they constitute likely targets of parasite induced selection.

Immunogenes – the target of selection

One way for a parasite to successfully infect a host is to evade recognition by host immunity. A host can only eliminate those parasites, which previously have been identified as non-self. In vertebrates self/non-self recognition is mainly regulated by genes of the major histocompatibility complex (MHC). Using three-spined sticklebacks and their naturally occurring macro-parasites as a model system, this thesis aims at investigating how the parasite fauna might interact with the host's MHC genes – especially if selection pressure is exerted on these recognition molecules.

Genes of the MHC are the most striking example for selectively maintained polymorphism in vertebrates. As early as 1966 a selective advantage of histocompatibility polymorphism was proposed (Clarke and Kirby 1966). Since then several lines of evidence support natural selection as cause for the maintenance of allelic polymorphism of MHC genes (Apanius et al. 1997). The crucial function of MHC molecules in initiating the adaptive immune response suggests that the ultimate agents of selection maintaining diversity are pathogens. Several studies demonstrated that single MHC alleles or MHC haplotypes are associated with either susceptibility or resistance to disease (for review see (Apanius et al. 1997)). The mechanism by which natural selection operates is, however, controversial, with negative frequency dependence and heterozygote advantage being discussed most often (Apanius et al. 1997; Penn and Potts 1999). These mechanisms are not mutually exclusive and a distinction between both modes of selection is difficult. Theoretical models and our

mechanistic understanding of how MHC molecules work imply that maximal diversity/heterozygosity does not confer the highest Darwinian fitness, because negative selection on T cell repertoire size narrows down the amount of detectable pathogens.
Olfactory based mate choice as function of MHC genotype has been identified in several species (for review see Penn and Potts 1999) additionally making MHC diversity a target for sexual selection. With regard to mate choice, the polymorphism displayed by MHC genes might serve two different evolutionary functions. First, it may signal genetic relatedness allowing a choosing partner to avoid mating with close kin and thereby inbreeding. Second, expression of secondary sexual traits signaling immunocompetence can correlate with individual MHC genotype (vonSchantz et al. 1996) or MHC genotypes can be assessed directly (Reusch et al. 2001a). If mechanisms exist that allow for maximizing offspring fitness by directly choosing the fittest MHC genotype, mate quality can be assessed without detours over secondary handicap signals. When the quality of offspring in terms of parasite resistance can be anticipated to some degree from the MHC immunogenetics of the chosen mate, hosts could win the upper hand in the arms race with their pathogens – providing a possible solution for the evolutionary paradox of the persistence of sexual reproduction.

Structure of the MHC region and MHC molecules

Not surprisingly, the MHC of humans is studied most intensively, but as we will show below, the architecture of MHC of in non-mammalian species may be markedly different. The MHC of humans covers an extensive, gene rich region of four megabases. Approximately 40% of the 224 identified genes are associated to functions of the immune system (MHC sequencing consortium, 1999). In other vertebrate groups the structure of the MHC region can look markedly different (Flajnik and Kasahara 2001). In chicken, for example, the gene cluster only contains a few genes and was therefore framed the “minimal essential MHC” (Kaufman et al. 1995). Other bird species such as songbirds have many classical and non-classical MHC genes in a single linkage group (Westerdahl et al. 2000). In bony fishes (*Teleostei*), on the other hand, genes are situated on different linkage groups (Flajnik and Kasahara 2001). Functional studies focused on the mammalian MHC, which structure is rather conserved. This might not be the general but rather the specialized case as in the majority of vertebrate species MHC genomic organization might be more complex. Common for all vertebrates is the outstanding role of MHC genes of class I and class II, since both gene families show

extensive nucleotide and allelic polymorphism. For example, the most polymorphic loci of the human MHC, also known as human leukocyte antigen (HLA), have 563 alleles at the HLA-*B* locus and 447 alleles at the HLA-*DRB1* locus (Robinson et al. 2003). Both genes present peptide antigens originating from proteolytic degradation of self or non-self proteins to T cells. Class I molecules are monomers with four extracellular domains ($\alpha_{1\text{-}3}$ and β_2m). Peptides, usually 9-mers, are bound in the peptide binding region (PBR) formed between the polar α_1 and α_2 domain. Class I molecules are expressed on nearly all cells types and present antigens to cytolytic CD 8+ T cells. Since an activated CD 8+ cell kills the cell, which is presenting a peptide with sufficient binding affinity, MHC class I serves surveillance of infection with intracellular parasites and occurrence of malign tumors.

Class II molecules are heterodimers of two peptide chains, the α and β chain encoded by A and B genes that often occur in tandem arrangement. The diversity of potential class II molecules is a quadratic function of the number of class II A and B tandems because combination of all alpha and beta chains to form a functional class II molecule is possible. The α and β chain both possess two extracellular domains. Peptides, in this case 9-12 mers, are bound in the pocket formed between the distal α_1 and β_1 domain. Class II molecules are only expressed on specialized antigen presenting cells (e.g. dentritic cells, B cells, macrophages) and peptides are in turn presented to CD 4+ T helper cells. Both classes of MHC genes have in common, that only certain anchor residues, which actually stick out into the binding pocket, are involved in binding peptides.

Shaping of T cell repertoire

The synchronous appearance of MHC genes and other keystone genes of the adaptive immune system (i.e. RAG genes) in cartilaginous fish, $540*10^6$ years ago (Kasahara et al. 2004) , hints to one of the primary functions of MHC genes, which is to control the correct targeting of the adaptive immune system. This quality control is essentially the discrimination between self and non-self with subsequent shaping and directing of the T cell repertoire. Out of the 10^{15} different thymocytes that can theoretically be created by exon shuffling (Davis and Bjorkman 1988), only 5% mature to CD4+ or CD8+ naïve T cells via positive and negative selection processes (Goldrath and Bevan 1999). These two opposite filters that shape a vertebrate’s T-cell repertoire. All cells that bind to

either a MHC class I or class II molecule in the thymus early in development with sufficient affinity are positively selected. Those thymocytes are then subjected to a second round of selection. Here, all cell lines above a critical affinity are deleted, as these would induce an autoimmune response once released to the periphery. The remaining naïve T cell repertoire will consist of medium self-affinity TCRs, which will preferentially bind foreign antigens presented by an MHC molecule in the periphery.

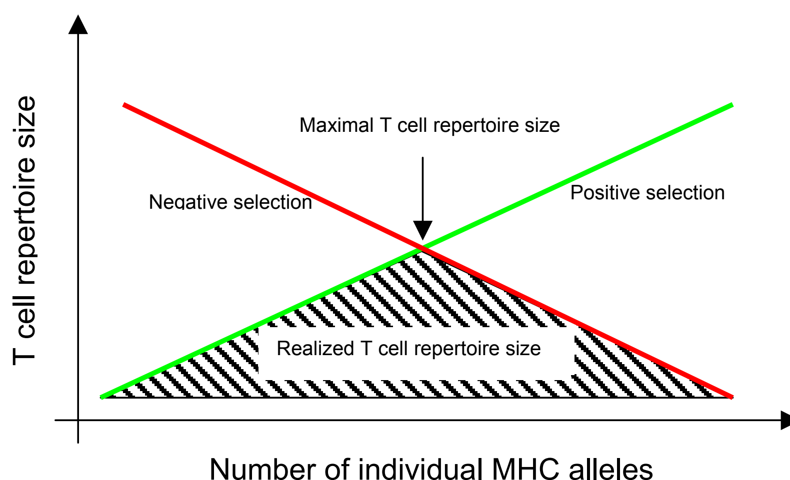


Figure 2: Balance between positive selection (green line) and negative selection (red line) forming the realized T cell repertoire size (hashed area beneath curves). Maximal diversity can be expected at intermediate levels of individual MHC diversity.

It follows that the T cell repertoire size is crucially dependent on the number of MHC molecules (Fig. 2), and thus, on MHC class I and II genes present. Any additional new MHC molecule will increase the number of positively selected T cell lines in the functional repertoire thereby enhancing the chance of detecting an intruding pathogen (Doherty and Zinkernagel 1975). An individual with higher individual MHC diversity (i.e. higher MHC heterozygosity) will then possess a selective advantage. On the other hand even in the periphery up to 85% of MHC molecules are loaded with peptides that are derived from endogenous self molecules (Chicz et al. 1993). However, negative selection inside the thymus will guarantee that all naïve T cells will only have a low affinity binding to these self-peptide-MHC complexes. Therefore, the balance between positive and negative selection theoretically leads to an optimal number of different MHC molecules (Nowak et al. 1992) or an optimal MHC heterozygosity (Apanius et al. 1997; Penn and Potts 1999) within individuals (Fig. 2). The absolute magnitude of optimal diversity is, however, matter of debate. While some authors conclude that the calculated optimal diversity would roughly correspond to the observed number of MHC alleles found in humans (Nowak et al. 1992), others argue that the individual MHC

diversity corresponding to the largest effective T cell repertoire is much higher (Borghans et al. 2003). This discrepancy stems from different relative weightings of positive and negative selection, as well as from uncertainties related to the magnitude of cross-reactivity. Borghans et al. (2003) view positive selection, which only selects about 3-5% of the vast number of thymocyte clones, as the main bottleneck for shaping the naïve repertoire. Even when one assumes negative selection to delete half of the previously positively selected clones the maximal T cell repertoire can be found at extremely high diversities of 10^3 different MHC alleles per individual (i.e. around 100 gene loci). However, it has to be noted that MHC restriction will not be absolute even if alloreactivity as well as self-MHC restriction are not high (Detours and Perelson 2000). With the more realistic assumptions of these models, the question arises why the realized MHC diversity is much lower in reality than expected, when a single duplication of any MHC locus will also enhance immunogenetic surveillance (Doherty and Zinkernagel 1975). This would in turn increase fitness by polylocism (i.e. duplication of loci) rather than polymorphism. Furthermore, while there are species possessing only single loci of MHC class I and class II (e.g. salmon (Langefors et al. 2001; Grimholt et al. 2003) or chicken (Kaufman et al. 1995), there seems to be an upper bound for the advantages conferred by polylocism. Polyploid populations of *Xenopus* seem to silence expression of MHC loci on duplicated chromosomes, which supports the notion that a too high copy number is indeed detrimental (Du Pasquier et al. 1989). The same seems to be true for some polyploid fish species. Lake Tana barbels *Barbus intermedius* (Dixon et al. 1996; Kruiswijk et al. 2004) and gynogenetic carps *Cyprinius carpio* (vanErp et al. 1996) seem to possess silencing mechanism as well, at least for MHC class II genes. These studies suggest that realistic optimal individual diversities in MHC molecules may be within realistic number found in vertebrates. Relative importance of class II silencing might be higher, because class II genes seem to be associated more frequently with autoimmune disease (Apanius et al. 1997). Also, the combinatorial diversity of class IIA and B chains leads to a faster increase of potential MHC II molecule diversity with increasing gene copy number.

Parasite infections: correlations and experiments

When natural selection favours diversity within MHC genes by overdominant selection individuals heterozygous at MHC loci should have a selective advantage. Already Doherty and Zinkernagel showed that the T cell response to virally infected cells is

enhanced in heterozygotes of MHC haplotypes of different specificities(Doherty and Zinkernagel 1975). The increase in immunological surveillance in heterozygotes provides an evolutionary advantage of MHC diversity. However, evidence for MHC heterozygote advantage so far has been ambiguous with some studies showing some form of fitness advantage in heterozygotes (Tab. 1), while others failed to do so. We argue here that this is most likely a consequence of the experimental design. Most studies focused on only a single pathogen species or strain. Within these studies MHC heterozygosity seems to be advantageous when highly variable pathogens were considered (e.g. HIV (Carrington et al. 1999)or other viruses (Thursz et al. 1995; Senseney et al. 2000; Arkush et al. 2002)). Most of the studies found associations between the pathogens and certain haplotypes or even single alleles and resistance is then inherited as a dominant trait (McClelland et al. 2003). These associations cover a wide range of host as well as pathogen taxa (for review see Apanius et al. 1997). Due to medical applicability taxonomic bias is shifted towards mammals (Bernatchez and Landry 2003) - especially humans where a lot of studies established correlative evidence for interactions between MHC genotype and course of infections with various pathogens. Among the first relevant associations shown in humans was the discovery of a common protective allele/haplotype against severe malaria in areas of high disease prevalence (Hill et al. 1991). The clearance of hepatitis viruses is also subject to presence of certain MHC class II alleles within the infected host (Thursz et al. 1995). One of the best studied examples is HIV, where beneficial and detrimental effects on disease progression are known for several MHC class I alleles (Carrington et al. 1999). Aside from viruses and microparasites, infection with macroparasitic worms can also be mediated by MHC genotype of the host. Severe forms of infection with *Echinococcus* tapeworms is for example associated with the HLA B8, DR3, DQ2 haplotype (Godot et al. 2000).

The complex structure of the mammalian MHC with many gene loci on a single linkage group makes the identification of single alleles causing resistance or susceptibility difficult. Therefore, strong associations between resistance and MHC genotype were predominantly found in species with rather simple structure of their MHC, as is the case in chicken suffering from infection with Marek's disease (Briles et al. 1977). Here, the presence of line-specific MHC alleles could account for an eight-fold increase in tumor incidence, thereby explaining a major proportion of the additive genetic variance in resistance between the selected lines. Other associations are reported in Atlantic salmon

Salmo salar (Langefors et al. 2001; Lohm et al. 2002; Grimholt et al. 2003), which represents a suitable non-mamalian model species. First of all, in teleost fish MHC class I and class II are not on the same linkage group (Flajnik and Kasahara 2001), which allows to tear apart the different effect contributed by class I and class II genes. Furthermore, salmon seem only to express single loci of class I and class II (the minimal essential bony fish MHC) additionally simplifying the genomic structure. Bacterial infection with *Aeromonas salmonicida* was reduced in the presence of certain MHC class II alleles (Langefors et al. 2001; Grimholt et al. 2003), which was independent of other genetic background genes (Lohm et al. 2002).

The primary research goal of above studies was to discover MHC haplotypes/alleles that confer resistance, yet ironically more alleles/haplotypes were found to be associated with susceptibility to disease (Jeffery and Bangham 2000). This is in line with theoretical predictions of host-parasite coevolution. Susceptibility is expected to be more common because fast evolution of the parasite is assumed to fuel the arms race between them and their hosts. For most pathogens it is valid to assume a higher evolutionary potential compared to that of the host because generation times are usually much shorter or effective population sizes of pathogen populations are larger. The human HLA-*A11* allele, for example, confers resistance to infection with Epstein-Barr-virus only in populations where the allele is rare. In populations with high frequency of this allele virus strains have fixed a mutation that prevents presentation of immuno-dominant epitopes by HLA-*A11* molecules (Decamposlima et al. 1993). While this shows that a fast evolving pathogen is able to adapt to host defense, it also demonstrates the frequency dependence of selection between parasites and hosts in the form of a rare allele advantage in the host population.

MHC heterozygote advantage

MHC mediated resistance to a single pathogen is inherited as a dominant trait (McClelland et al. 2000; Penn et al. 2002). This means that there will be no differences in susceptibility between a homozygote MHC allele or haplotype and a heterozygote carrying the focal allele plus a different one. Therefore, heterozygote advantage is difficult to detect in single pathogen challenges (Apanius et al. 1997).

One of the first reports of MHC heterozygote advantage in the face of a multi-parasite challenge comes from mole rats *Spalax ehrenbergi*, where population-wide infection

rates with ectoparasitic mites and endoparasitic helminths correlate with average MHC class II heterozygosity (Nevo and Beiles 1992).

If MHC heterozygote adavantage occurs in single or multiple pathogen challenges, a general heterozygote advantage has to be ruled out since MHC heterozygosity could correlate with genome wide heterozygosty. Despite the detrimental effects of deleterious recessive alleles leading to general heterozygote advantage, only few studies could rule out background genetic variability as a possible source for increased fitness (Carrington et al. 1999). However, this objection mainly applies to correlative studies from natural populations. Most laboratory studies circumvent this problem by mixing the genetic background in F2 backcrosses. Using such an experimental population Penn et al. (Penn et al. 2002) could show that MHC heterozygotes had a selective advantage when challenged by multiple strains of bacteria. When challenged with *Listeria* and multiple strains of *Salmonella* MHC heterozygotes had lower pathogen loads as well and suffered less from weight loss than homozygotes. In another elegant experiment McClelland et al.(2003) paired mice homozygous for haplotype $H2^q$ with homozygotes for the $H2^b$ haplotype. $H2^q$ confers resistance against *Salmonella* infection but is associated with an increased susceptibility for Theiler's virus. The $H2^b$-haplotype shows exactly a reversed susceptibility profile for the two pathogens. Analysis of F2-segregants showed clearly that heterozygotes with this genetic background had the lowest combined parasite load. However, MHC heterozygotes are not generally more resistant as specific associations between the haplotype and the disease are needed. Individuals carrying different haplotypes showing similar susceptibility profiles did not display increased resistance. This set of experiments shows that even alleles associated with susceptibility to one pathogen can selectively be maintained when they simultaneously confer resistance against other pathogens.

MHC mediated mate choice in support of parasite resistance?

Mating patterns of MHC genes are often not random and usually reflect disassortative mating (Penn and Potts 1999). Therefore, mechanisms for MHC mediated mate choice are likely to exist and contribute to the polymorphism of MHC genes. However, if heterozygosity or optimality is advantageous, mate choice would then also maximize offspring fitness by reflecting the pattern of parasite mediated selection. MHC dependent mate choice has been demonstrated for example in the case of mice (Yamazaki et al. 1978; Potts et al. 1991), fish (e.g. salmon (Landry et al. 2001),

sticklebacks (Reusch et al. 2001a)), birds (vonSchantz et al. 1996) and humans (Wedekind and Furi 1997). Several models of the evolution of MHC dependent mate choice have been developed, which need not to be pathogen driven (for review see Penn and Potts 1999). However, since disassortative mate choice in mice leads to MHC heterozygous offspring sexual selection would go hand in hand with pathogen driven natural selection – at least in the cases presented above, which suggest that in multiple simultaneous infection treatments MHC heterozygosity increases parasite resistance (Penn et al. 2002; McClelland et al. 2003). In the case of salmon MHC mediated mate choice is dependent on the actual differences in amino acid composition between alleles (Landry et al. 2001). This makes intuitive sense because the higher the difference between alleles is, the more likely it becomes that the two alleles also bind peptides derived from different pathogens. However, it remains to be shown that this also has an effect in terms of parasite resistance.

Additionally, sexual selection is not solely mate choice, but can also be cryptic by means of egg-sperm interactions. Fertilization of the egg could depend on the MHC genotype of the egg and of the fertilizing sperm. Only a limited number of possible haplotype combinations would then lead to successful fertilization. Mice seem to possess such a mechanism. At least when mothers are challenged with viral infections they tend to produce more MHC heterozygous offspring (Rulicke et al. 1998).

MHC dependent mate choice in sticklebacks utilizes more complex mechanisms (Reusch et al. 2001a). The female chooses mates by evaluating the number of MHC alleles the male does not share with herself. If offered water originating from two male tanks, the female prefers the smell of the male, that is complementing her own alleles best to produce offspring with an intermediate number of alleles (Reusch et al. 2001a; Aeschlimann et al. 2003), while those leading on average to higher or lower numbers, are deselected. Evidently, if this complex behaviour evolved to efficiently defend against parasites, the strength of parasite induced selection must have been strong. To elaborate the role of MHC class IIB genes for parasite resistance in three-spined sticklebacks is therefore one of the main objectives of this thesis.

Genotyping of MHC class II in sticklebacks

To enable a fast and efficient screening of MHC class IIB polymorphism of a large number of individuals, procedures involving the cloning of PCR products are of little

practical use, because they are extremely time consuming. Especially when it comes to

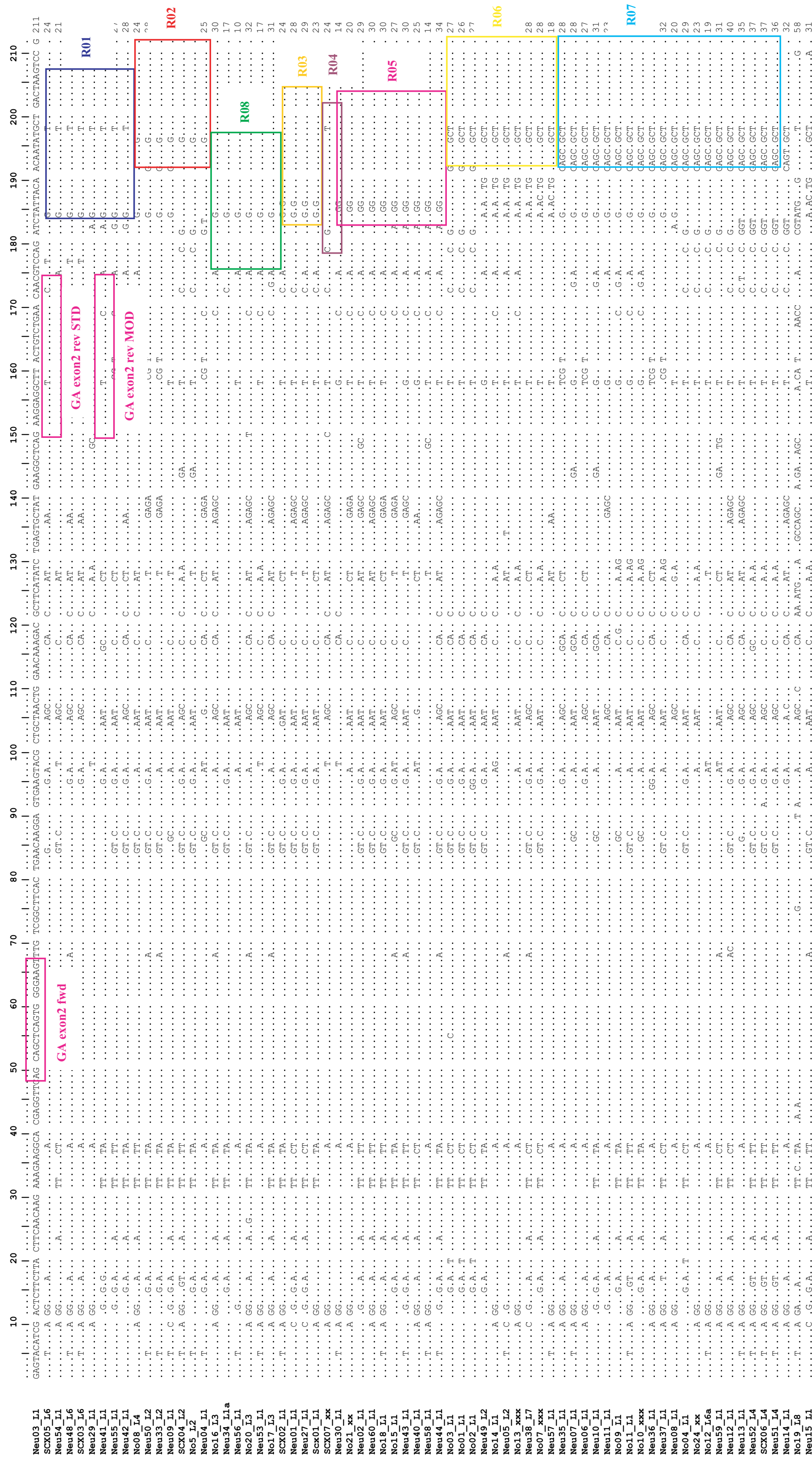


Fig. 3: Aligment of stickleback MHC class IIB exon 2 sequences. Boxes indicate the binding sites of the primers used for PCR amplifiacation. GA exon2 rev STD and GA exon2 rev MOD occur multiple times but are only marked once. Motif specific reverse primers (R01 – R08) all use GA 11 as forward primer. GA 11 can be found on the 5' end of all sequences and is therefore left out.

duplictated genes like the MHC class IIB cloning and sequencing work becomes tedious. Furthermore, MHC class IIB loci of sticklebacks have been duplicated relatively recently (Reusch et al. 2004) and the flanking regions of the immunologically important exon 2 are too similar to design locus specific primers. We therefore used a genotyping method with which different sequences of MHC class IIB genes can rapidly be discriminated – i.e. single stranded conformation polymorphism (SSCP). SSCP allows single strands of PCR products to fold back onto itself to form a sequence-specific secondary structure, which can in turn be separated by capillary electrophoresis in a native, non-denaturing polymer (Fig. 4). We applied two strategies: The first one amplified a stretch of 120 bp including the most polymorphic part of the peptide binding region (PBR, Binz et al. 2001). To do this, two PCRs were performed both using “GA exon2 fwd” as forward primer but using two different reverse primers (i.e. GA exon2 rev STD and GA exon2 rev MOD, Fig. 3). By employing this method roughly 80% of alleles were detected.

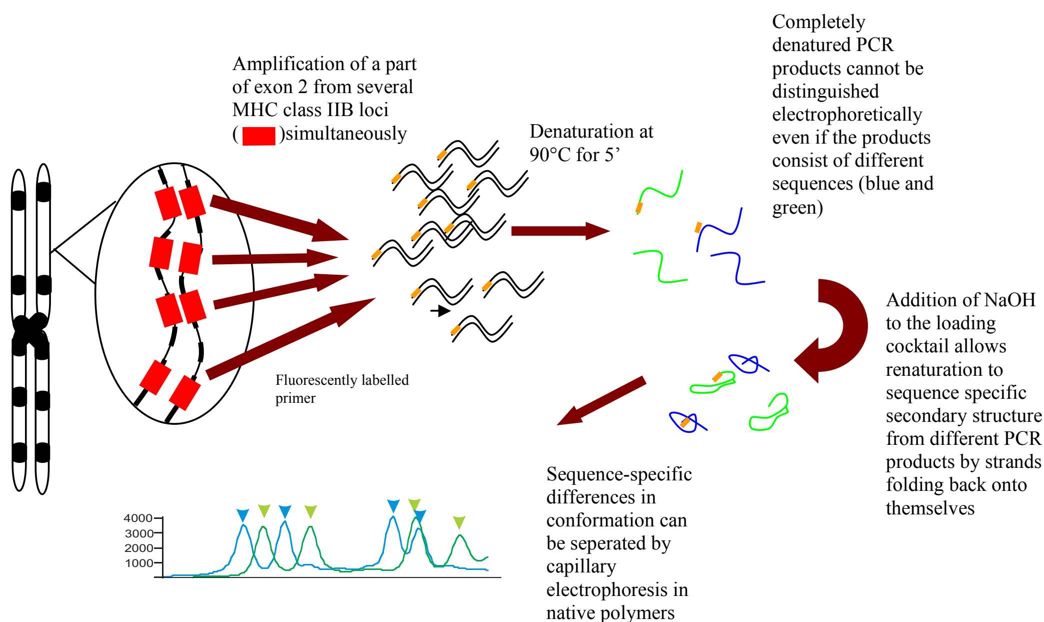


Figure 4: Schematic representation of MHC class IIB genotyping in sticklebacks by single stranded conformation polymorphism (SSCP). PCR products of different sequences can be separated by electrophoresis when sequences are allowed to fold back onto themselves and form a sequence-specific secondary structure.

To increase the resolution of the genotyping we also applied a second strategy. From a set of 69 sequences eight primer combinations were designed. All primer combinations contained the same forward primer (GA11, Sato et al. 1998), which was used to

generate the sequences in the first place. The eight reverse primers (R01 – R08) were motif-specific and placed at the end of the exon (Fig. 3). This increased the chances of picking up most of the polymorphisms while at the same time increasing the power to differentiate between two distinct sequences, because less PCR products are generated per primer pair in individuals as well as in total.

Parasites of the three-spined stickleback

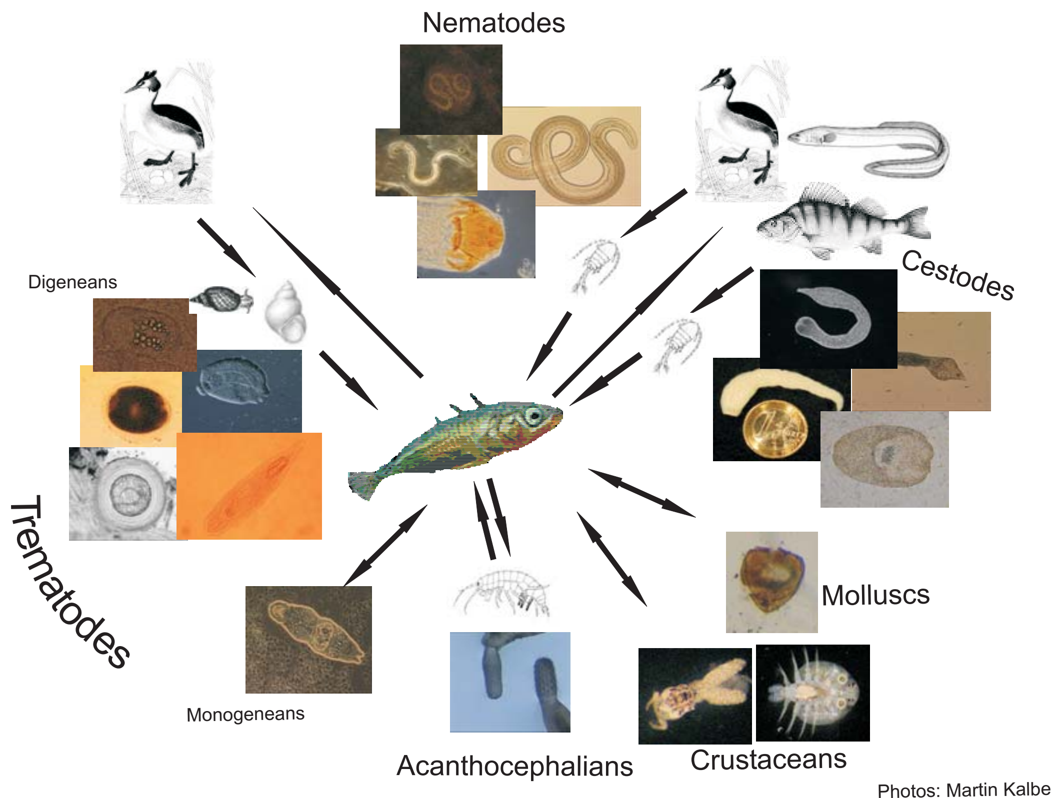


Figure 5: The three-spined stickleback is part of the life cycle of many macroparasite taxa. Cestodes and nematodes rely mainly on copepods as first intermediate hosts and use either predatory fish or fish eating birds as final hosts. First intermediate hosts of digenean trematodes are always molluscs (snails and bivalves) and final hosts are most often fish eating birds. Sticklebacks are also final host for Acantocephalians with gammarids as intermediate hosts and are host to several taxa with direct transmission (i.e. monogeneans *Gyrodactylus gasterostei*, crustaceans *Ergasilus spec.* and *Argulus foliaceus* and Glochidia molluscs.

Three-spined sticklebacks are hosts for a wide range of parasites. Besides being struck by infections of microparasitic bacteria, viruses, fungi and protozoans, sticklebacks also play an important role, either as intermediate of definitive host in the life cycle of several macro-parasites (Fig. 5, Marcogliese 1994). This study focuses on macro-parasites, because these tend not to reproduce within hosts (monogeneans are an

exception for example) and are therefore easier to quantify and handle experimentally. Furthermore, all macroparasites are extracellular and an interaction with MHC class II genes is more likely to be involved in an immune response. Parasites harm their hosts in many ways, the most common being energy drain or behavioural manipulation. There is virtually not a single fish, which is parasite free, while most fish suffer from multiple species infection. Therefore, the impact of parasites on fitness is likely to be profound.
So far, more than 30 species of parasites were found in sticklebacks from northern Germany, covering most of the important macroparasitic taxonomic groups (Kalbe et al. 2002).

Nematodes

Figure 6: Nematode parasites of the three-spined stickleback. From left to right: *Anguillicola crassus* in the wall of a swim bladder, head of *Camalanus lacustris*, *Contracaecum spec.* and liver cyst of *Raphidascaris acus*.

Photos: M. Kalbe

Parasites of the taxon Nematoda were represented by *Anguillicola crassus*, *Contracaecum spec*., *Camallanus lacustris* and a *Raphidascaris acus* (Fig. 6). *A. crassus* was recently introduced to Northern Germany, where it causes considerable harm to populations of its definitive host, the European eel *Anguilla anguilla* (Barus and Prokes 1996; Wurtz et al. 1998). The life cycle includes a copepod as first intermediate host before transmission to the final hosts proceeds either directly or by paratenic hosts like the three-spined stickleback. Even though sticklebacks are no obligatory part of the life cycle infections are not rare (Kalbe et al. 2002) and damage caused to the wall of the swim bladder can handicap fish considerably (Wurtz and Taraschewski 2000). *C. lacustris* is also transmitted by copepod first intermediate hosts and the final host is perch *Percus fluvalis*. The worm attaches itself to the gut wall and lives from blood feeding, which causes energy drain and tissue damage (Lodes and Yoshino 1985). *Contracaecum spec*. encysts itself into all types of soft tissue, preferentially gut tissue, while *Raphidascaris acus* forms cysts in the liver or the intestine.

Trematodes

Figure 7: Digenean trematodes parasitising three-spined sticklebacks. From left to right: cyst of *Cyathocotyle prussica*, metacercaria of *Diplostomum pseudospathacaeum*, eye lens infected with *D. pseudospathacaeum* metacercariae, *Echinochasmus spec.* metacercaria, gill arch of a stickleback infected by *Echinocasmus spec.* metacercariae, encysted metacercaria of *Apatemon cobitidis*, *Tylodelphis clavata***.**

Photos: M. Kalbe

Digenean trematode species were represented by *Diplostomum pseudospathacaeum*, *Cyathocotyle prussica, Apatemon cobitidis, Tylodelphis clavata* and *Echinochasmus spec*. (Fig. 7). These parasites need a mollusc first intermediate host. Since sticklebacks are only second intermediate hosts a bird needs to eat an infected fish to complete the parasite's life cycle. Therefore most of the digenean parasites of sticklebacks try to increase their transmission probability by lowering the host's flight capabilities. *Diplostomum pseudospathacaeum* cercariae are released by *Lynmnaea stagnalis* snails and actively search for their second intermediate host. Once a fish is reached they from metacerariae in the eye lens impeding host vision (Crowden and Broom 1980; Owen et al. 1993). Metacercariae of *Tylodelphis clavata* can be found within the glass body of the eye and *Apatemon cobitidis* might constrain vision by attaching to the optical nerve. *Cyathocotyle prussica* often forms cysts in muscle tissue, which might decrease functionality of the musle. *Echinocasmus* encysts in the gills of a fish, which might decrease the efficiency of oxygen transport and thereby limits flight distance. One species of monogenean trematodes was found (i.e. *Gyrodactylus gasterostei,* Fig. 8, identification by Joost Raeyemakers, pers. com.). Due to their reproduction on the host, densities on single fish can reach up to more than 400 worms. These worms feed on skin and mucus. Therefore, the chance of getting a secondary bacterial infection is elevated and fish induce an immune response to eliminate the worms (Harris et al. 1998; Buchmann 1999).

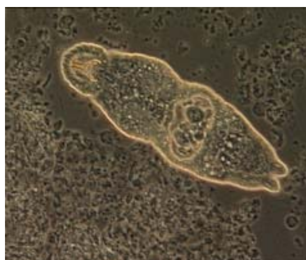


Figure 8:*Gyrodactylus gasterostei*

Photo: M. Kalbe

Cestodes

Figure 9: Cestode parasites of three-spined sticklebacks. From left to right: *Schistocephalus solidus* in relation to a 1€-coin, *Valipora campylancristrota,* gall bladder heavily infected by *Valipora campylancristrota*, *Protecephalus filicollis* and *Paradilepis scolecina.*

Photos: M. Kalbe

Cestodes parasitizing three-spined sticklebacks were *Valipora campylancristrota*, *Paradilepis scolecina* and *Proteocephalus filicollis* (Fig. 9). These cestodes have similar life-cycles with copepods as first intermediate hosts and fish eating birds or predatory fish as definitive host. The intestinal *P. filicollis* uses sticklebacks as final hosts and has only 2-step life cycle. *V. campylancristrota*. can be found in the gall bladder, while *P. scolecina* is also an inhabitant of the gut.

Other taxa

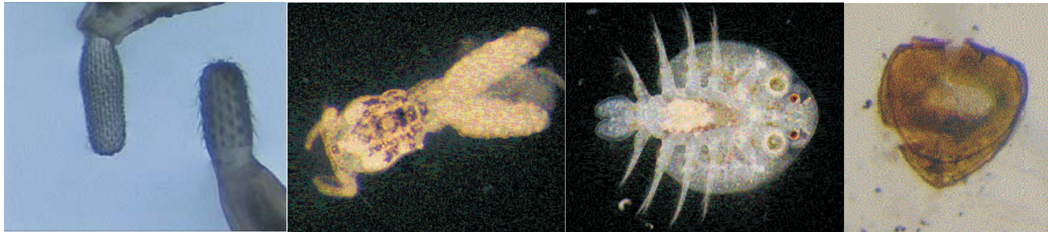


Figure 10: Other macroparasite taxa infecting three-spined sticklebacks. From left to right: Acantocephalians *Acantocepahalus lucii* and *A. clavula*, crustaceans *Ergasilus spec.* and *Argulus foliacaeus*, mollusc *Glochidia.*

Photos: M. Kalbe

Fig. 10 shows parasites of other taxa. These include acantocephalians attaching with their scolex to the gut wall, crustaceans of the genus *Ergasilus* parasitizing gills and *A. foliacaeus* parasitizing the skin, along with a parasitic Glochidia (parasitic stages of freshwater bivalves). These species were encountered relatively rarely and can be regarded to be of minor importance.

Thesis outline

This thesis is divided into five chapters. Each chapter is an independent study addressing different aspects of parasite induced selection on MHC class IIB genes in three-spined sticklebacks. This outline gives a short overview of the motivation for the single experiments.

Chapter I

The first chapter describes the geographical pattern of allelic variation in MHC class IIB genes of different populations from Schleswig-Holstein. Individuals from eight populations were analysed, comprising three different habitat types with markedly different ecological conditions, i.e. lakes, rivers and estuaries. Replicated sampling should reveal how the variation in MHC class IIB is connected to parasite prevalence in individuals as well as on the population level. Furthermore, I discuss to what extent the observed variation could be explained by parasite induced natural selection opposed to other non-selective processes changing genotype frequencies, particularly genetic drift and gene flow. To differentiate between selection on MHC genes and neutral processes, selectively neutral markers (microsatellites) were used. If parasites exert selection pressure on MHC class IIB genes, one would expect to find more allelic diversity of MHC class IIB genes in populations with greater parasite diversity while no such difference should be found for the neutral loci. This should also be reflected in individuals, the ultimate unit of selection. A high allelic diversity can only be maintained in populations when an individual carrying a higher number of alleles is protected better against infection by a diverse parasite fauna (i.e. MHC heterozygote advantage).

Chapter II

The correlative results presented in Chapter I suggest that individuals with an intermediate individual MHC class IIB diversity were on average infected with less parasite species than individuals with an allelic diversity above or below this presumed optimum. We decided to repeat the results from the field in a controlled laboratory experiment. To do so, we bred six stickleback families whose MHC class IIB genotypes ranged from low to high individual diversity, always including one genotype close to the previously determined optimum. In controlled infection experiments, these fish were

exposed to the three parasite species, which seem to be most important in the population where the parents of the fish stemmed from. The use of within-family analysis also enabled us to narrow down genetic effects to the MHC class II region, because genes from other linkage groups will be inherited independently from any given MHC class II haplotype. We hypothesized that fish with an intermediate number of MHC class IIB alleles would show the lowest parasite burden.

Chapter III

Allelic variants with mutations in exonic DNA sequences that get translated to proteins are not the only source of genetic variation for natural selection to work on. It is not only important which allelic variant or gene copy is present but also its expression level and target tissue of expression. By using the experimentally infected fish from Chapter II and inbred lines derived from these, we wanted to look at MHC class IIB expression patterns. We developed a real time PCR assay measuring relative expression of MHC class IIB compared to the constitutively expressed house keeping gene β actin. We wanted to address two aspects with this study: First, we determined whether exposure to parasites influenced the MHC expression levels, hypothesizing that exposure should excite the immune system and therefore increase expression levels. Secondly, we wanted to look for a genetic basis in MHC expression patterns, which can be revealed by differences between families. Finding these differences also in the daughter generation would reveal a heritable component of MHC expression control. Due to the importance of individual MHC diversity for parasite defence, we were also interested how this trait would influence the expression level.

Chapter IV

The correlative finding of an immunogenetic optimum in wild caught fish identified in Chapter I and its later confirmation in a controlled experiment (Chapter II) have both advantages and draw-backs. In a field study usually none of the variables of interest are controlled giving rise to the possibility of arbitrary random correlations. Experiments, on the other hand, can only mimic the natural situation to a certain degree and tend to exaggerate the investigated effect. Therefore, we wanted to combine both approaches by exposing lab bred fish from 14 families to their natural parasite fauna *in situ* by using enclosure cages in the natural environment. Families were chosen applying the same criteria as in Chapter II with the additional inclusion of families, which did not show

variation in the number of MHC alleles. This approach also opens up the possibility for mortality, because conditions in the field are likely to be more harsh than the benign conditions in the lab. If parasites have a profound effect on fitness one could expect that the combination of several stress sources including parasitism might lead to elevated mortality. Following from our earlier results (Chapter I, II), we would expect that immunologically optimal fish would survive better than their suboptimal kin.

Chapter V

We could convincingly show that MHC heterozygote advantage could be one mechanism of the balancing selection maintaining MHC class IIB polymorphism. However, the Red-Queen theory of host-parasite co-evolution states that due to tracking of common host genotypes by parasites, fitness contributed by single alleles should fluctuate over time. The final chapter addresses this question by looking for frequency fluctuations of MHC class IIB alleles, which are coupled to frequency changes in parasite prevalence. We used three populations and sampled these over a period of three years, which roughly equates to three generations of the stickleback hosts. To find antagonistic oscillations between MHC class IIB alleles and parasites 25 fish per year and location were dissected and genotyped. Similar to the approach in Chapter I we also wanted to exclude non-selective causes for the observed pattern and analogous to Chapter I we compared patterns at MHC class IIB with that of neutral markers. We predict that fluctuations over time should be stronger in MHC class IIB alleles if they are associated to infection/resistance to parasitic infection.

Sampling locations

Extreme sticklebacking

Photo: Martin Kalbe

CHAPTER I

Multiple parasites are driving Major Histocompatibility Complex (MHC) polymorphism in the wild

Abstract

Parasite mediated selection may result in arms races between host defence and parasite virulence. In particular, simultaneous infections from multiple parasite species should cause diversification (i. e. balancing selection) in resistance genes both at the population and the individual level. Here, we tested these ideas in highly polymorphic Major Histocompatibility Complex (MHC) genes from three-spined sticklebacks (*Gasterosteus aculeatus* L.). In eight natural populations, parasite diversity (15 different species), and MHC class II*B* diversity varied strongly between habitat types (lakes vs rivers vs estuaries) with lowest values in rivers. Partial correlation analysis revealed an influence of parasite diversity on MHC class II*B* variation while general genetic diversity assessed at 7 microsatellite loci was not significantly correlated with parasite diversity. Within individual fish, intermediate, rather than maximal allele numbers were associated with minimal parasite load, supporting theoretical models of self-reactive T-cell elimination. The optimal individual diversity matched those values female fish try to achieve in their offspring by mate choice. We thus present correlative evidence supporting the 'allele counting' strategy for optimising the immunocompetence in stickleback offspring.

Introduction

Hosts and parasites often engage in arms races (Van Valen 1973). These associations should result in close correlations between virulence traits of pathogen strains (or species) and the host's resistance genes, finally leading to diversification and balancing selection on both, virulence and resistance genes. The Major Histocompatibility Complex (MHC) of vertebrates is the prime example for resistance genes with extensive genetic polymorphism attributed to balancing selection (Klein 1986). MHC-gene encoded proteins present antigens to T-lymphocytes and initiate specific immune responses. An excess of replacement over silent nucleotide substitutions in Antigen Binding Regions (ABR) is strong evidence for balancing selection (Hughes and Nei 1988). The crucial role of MHC molecules in antigen presentation also suggests pathogens as selective agents, but support for this hypothesis is limited and indirect (Hughes and Nei 1988). A series of studies found associations between certain MHC alleles and infection with single pathogens (Hill et al. 1991; Thursz et al. 1995; Paterson et al. 1998; Godot et al. 2000; Langefors et al. 2001; Meyer and Thomson 2001), supporting the notion of direct selection on MHC alleles by pathogens. While in line with hypotheses on explaining MHC polymorphism, these findings are not sufficient to explain the extreme diversity of these genes. This is because any mechanism of balancing selection explaining MHC polymorphism with a single infectious agent would require extreme dynamics of host-parasite co-evolution (Peters and Lively 1999), which seems to be unlikely considering the stable distributions of macroparasites (Shaw et al. 1998). Rather, numerous pairs of single parasite-resistance-allele associations will result in diversifying selection on MHC alleles within host populations (Apanius et al. 1997). This is particularly relevant when taking into account that the majority of animal species are parasites (Windsor 1998), and that most animals suffer from multiple infections simultaneously. Surprisingly, we are not aware of any study examining the correlation between diversity of local parasite communities and MHC polymorphism.

Consequently, the goal of this study is to cover the full range of naturally occurring macroparasites in wild populations of three-spined sticklebacks (*Gasterosteus aculeatus* L.) for explaining MHC diversity. We predict that local populations exposed to a more diverse array of parasites will be more diverse in terms of their MHC genes. We focus here on MHC class II genes because they are most critical for triggering the immune response against non-viral, extra-cellular antigens (Hughes and Yeager 1998).

Moreover, some MHC class II haplotypes have been associated with clinic severity of cestode infections in humans (Godot et al. 2000) and infection with intestinal nematodes in a ruminant population (Paterson et al., 1998) indicating functional significance of MHC class II genes in defense against macroparasites. Within class II genes, the second exon of the β chain was selected because it composes parts of the functional important peptide binding groove, and has been shown to be the most polymorphic part in many class II genes (Ohta 1998; Hughes 1999).

In host-parasite associations, the target of selection is the individual. Evidently, the diversity among single genotypes is necessary to explain population-wide polymorphism (Doherty and Zinkernagel 1975; McClelland et al. 2000; Penn et al. 2002). This is particularly relevant when the genomic architecture of the MHC region is considered. There are only few species expressing only one MHC class II*B*-locus (such as salmon *Salmo salar* L., (Langefors et al. 1998), or having very compact regions (chicken *Gallus gallus* L., (Kaufman and Salomonsen 1997). In most other species, however, the MHC covers a extensive region of up to 4 Mbp and most loci of class I and class II are duplicated (Klein 1986). In particular the functionally important antigen-binding region (ABR) of MHC class II β chain has multiple expressed gene products in humans and other species (Swarbrick and Crawford 1997; Málaga-Trillo et al. 1998; McConnell et al. 1998; Hughes 1999). Assuming functional equivalency of these duplicated genes, individuals may thus posses between one (all loci homozygous with functionally similar copies at all loci) and two times the number of loci different alleles. Given that three-spined sticklebacks possess up to six MHC-class II*B*-loci (Sato et al. 1998; Reusch et al. 2001a) high levels of diversity can be created within individuals.

However, maximum individual diversity in MHC genes may not be beneficial. On theoretical grounds, Nowak et al.(1992) suggested an optimal, intermediate MHC. A high diversity at MHC class II*B* genes not only allows presentation of more antigens. As a negative consequence too many MHC variants will also result in presentation of more self-peptides, with subsequent elimination of parts of the total T-cell receptor lines per MHC-allele during ontogeny (Mason 2001). This process is exacerbated in class II molecules because they are heterodimers. Consequently, a combination of α and β chain will result in quadratic increase of functional class II molecules and T-cell elimination. Balancing selection would then outweigh diversifying selection and favour

an optimal number of MHC alleles. Additionally, recent findings of Reusch et al. (2001a) and Aeschlimann et al. (Reusch et al. 2001a; Aeschlimann et al. 2003) suggest that 'allele counting', a strategy of sexual selection, is also favouring intermediate MHC-diversity in offspring. Female fish select males with more alleles when they have less alleles than the population average themselves, while they tend to select males with lower individual MHC diversity when they have more MHC class II*B* alleles than the population average (Reusch et al. 2001a). Assuming an adaptive value of such a sophisticated choice mechanism, one possible cause maximizing the offspring's fitness may be an optimal MHC-diversity with lowest parasite burden.

Accordingly, we predict, in line with Nowak et al.'s (1992) theoretical prediction, that individual parasite load is lowest at an intermediate individual diversity of MHC class II*B* genes. We test these predictions in a survey of parasite infections in wild caught sticklebacks from 8 populations, and correlated their parasite load with MHC class II*B* diversity in individuals as well as populations.

Material and methods

Sampling design and parasite screening

Three- spined sticklebacks (*Gasterosteus aculeatus* L.) were caught during three periods (September, November, March) from eight water bodies (lakes and rivers) belonging to three catchments in northern Germany (Tab. I-1). Twenty-five fish of each catch were screened for parasite infections under a dissecting microscope. These fish harbored a total of 15 parasite species: **Ciliata:** *Trichodina spec., Apiosoma spec.;* **Microsporidia:** *Glugea anomala;* **Crustacea:** *Argulus foliaceus.;* **Monogenea:** *Gyrodactylus spec.;* **Digenea:** *Diplostomum pseudospathaceum*, *Echinochiasmus spec. (?)*, *Cyathocotyle prussica;* **Cestoda:** *Proteocephalus filicollis, Valipora campylancristrota;* **Acanthocephala:** *Acanthocephalus lucii, Acanthocephalus clavula;* **Nematoda:** *Camallanus lacustris.*, *Contracaecum spec.*, *Anguillicola crassus*. The diversity of the local parasite community was expressed as Simpson's diversity index *D* using following formula:

$$D = \frac{1}{\sum_{i=1}^{S} P_i^2}$$

where *S* is the number of species entering the index and P_i the relative abundance of species *i* to the total abundance of parasites.

Further information on populations and parasites can be obtained from Kalbe et al. (Kalbe et al. 2002).

Characterisation of MHC class II*B* variation

Immunogenetic variation was assessed in a larger sample of 1017 fish, which comprised the random subsample of 434 individuals subjected to dissection and parasite screening. Characterisation of MHC class II*B* genes involved an amplification of a 124 bp sequence internal to the Antigen Binding Region (ABR) of the exon 2 of MHC class II heavy β chain genes (Binz et al. 2001) with PCR reactions using two different combinations of primer pairs (Reusch et al. 2001a). As primers differed in two bases each pair amplifies a unique subset of MHC class IIB sequences. To identify different sequences we separated the fluorescently labelled PCR-products using Single Stranded Conformation Polymorphism (SSCP) on an ABI 310 Capillary Sequencer (Applied Biosystems, Weiterstadt, Germany). For simplicity, we refer to different class II*B* sequences as being alleles although they stem from different loci. Only those of the 434 dissected fish with unambiguous PCR products in both reactions were included in further analyses, finally leading to a sample size of n = 299. Both primer pairs amplify ≈80% of all present alleles (Reusch et al. 2001a). Additionally, we might have missed a certain proportion of the variation within the remaining base pairs of the whole exon. However, Reusch & Wegner (T. B. H. Reusch & K. M. Wegner, unpublished) found that in 56 more completely sequenced exons (211 base pairs) only three sequences were identical in the part of the exon included into the genotyping method used here, meaning that 95% of variation is covered by our typing method. Furthermore, as our null-hypotheses can only be rejected when finding variation, any lack in resolution of our genotyping technique is always conservative with respect to our hypotheses. Due to presumably up to six duplications of the β chain loci (Sato et al. 1998) each primer amplified on average more than two alleles. In our data set the first primer pair amplified 2.78 (± 0.96) while the second primer pair amplified 3.01 (± 0.99) alleles per individual, resulting in 5.83 (± 1.55) alleles/individual ranging from a minimum of 2 to a maximum of 9 in total. Such a range in identifiable allele numbers can either be explained by inter-individual variance in the number of loci present, a phenomenon

previously described for teleost fish (Málaga-Trillo et al. 1998), or by recent duplications bearing functionally equivalent sequences. Work on the development of single locus primers is in progress but difficult due to the recency of locus duplications (Reusch et al. 2004). The amplification of PCR products from duplicated genes may on the other hand overestimate the functional variability, as not all copies of the gene necessarily get expressed. However, from preliminary expression studies (K. M. Wegner, T. B. H. Reusch & T. Boehm, unpublished) revealed that most of the sequences covered by our genotyping are expressed.

Table I-1: Sampled three-spined stickleback (*Gasterosteus aculeatus*) populations, their parasite diversity as Simpson's *D*, MHC class II*B* and microsatellite diversity for pooled populations and single time points. Trammer See samples from November 2000 and March 2001 were omitted due to low number of fish caught. nd = not determined

Population			Dieksee	Kl. Plöner See	Trammer See	Vierer See	Schwale	Rönnau	Söhren	Wedeler Au
Catchment*			SC	SC	SC	SC	ST	TR	SC	EL
Habitat			Lake	Lake	Lake	Lake	River	River	River	Estuary
Parasite diversity	Simpson's D	Total	3.761	1.366	3.929	3.421	1.101	1.000	1.346	0.681
		Sept2000	3.907	2.054	3.929	4.449	1.020	1.000	1.346	0.526
		Nov2000	2.276	1.359	nd	3.338	1.100	nd	nd	0.727
		Mar2001	2.479	2.217	nd	2.375	1.003	nd	nd	0.790
MHC	Total alleles	Mean	23	24	25	23.667	13	13	11	25
		Sept2000	23	25	25	25	13	13	11	nd
		Nov2000	23	24	nd	24	13	nd	nd	28
		Mar2001	23	23	nd	22	13	nd	nd	22
Microsatellites	Alleles / locus	Total	6.86	7.14	8.57	10.29	7.14	nd	5.00	13.29
		Sept2000	5.86	5.57	7.29	6.43	6.00	nd	5.00	10.57
		Nov2000	5.00	7.00	nd	6.43	6.29	nd	nd	10.71
		Mar2001	5.29	5.14	nd	6.29	4.86	nd	nd	8.57

* SC = Schwentine, ST = St|r, TR = Trave, EL = Elbe

Microsatellite genotyping

Genome-wide variability has previously been shown to explain most of MHC population structure and diversity (Boyce et al. 1997; Hedrick et al. 2001b), at least in endangered species with comparatively small population sizes. Hence, we tried to estimate the role of selectively neutral processes (genetic drift) for explaining MHC polymorphism using microsatellite markers. Accordingly, we typed the majority of individuals comprised in the MHC genotyping (i.e. n = 920) at seven polymorphic

microsatellite loci (Largiader et al. 1999) under conditions for PCR amplification described by Reusch et al. (Reusch et al. 2001b).

Statistical methods

We compared the total allele diversity of MHC with the genome-wide variability (i.e. all alleles present for MHC and alleles/locus for microsatellites, respectively) and with parasite diversity at the population level. In two of the three linear regressions test of normality or homogeneity of variance failed. Therefore, we used non-parametric Spearman's rank correlation coefficients ρ. To determine the relative importance of either pairwise correlation the other variable was statistically kept constant by using partial correlations. As the individual data did not violate assumptions of normality and homogeneity of variance, the correlation among individual MHC-diversity and parasite load was estimated by ordinary least square regression. Individual parasite load varied strongly between habitats. To reduce variance introduced by this factor, we used residuals around population means as an indicator of individual parasite load. Confidence limits for the minimum of the quadratic polynomial were obtained by bootstrapping the individual data set 1000 times. Quadratic polynomials were fitted to each bootstrap replicate and minima were calculated.

Results

Parasite versus MHC-diversity in populations

In the study populations, we found high levels of diversity ranging from 13 to 28 different MHC class II*B* alleles (Tab. I-1). This estimate was rather conservative because only 80% of alleles will be detected by our genotyping method and some sequences cannot be resolved by SSCP (Reusch et al. 2001a). Populations being exposed to a wider array of macroparasites tended to be more diverse in terms of their MHC class II*B* genes (partial Spearman's rank correlation = 0.808, $P < 0.001$, Fig. I-1a). As a null model, any genetic diversity should be largely governed by population size and structure (Nei et al. 1975; Kimura 1983). Accordingly, we found a marginally significant correlation in the number of MHC class II*B* alleles and microsatellite alleles per locus at the population level (partial Spearman's rank correlation: 0.495, $P = 0.052$, Fig. I-1b). On the other hand, we found no correlation of parasite diversity with genome-wide variability (partial Spearman's rank correlation = -0.248, n.s., Fig. I-1c).

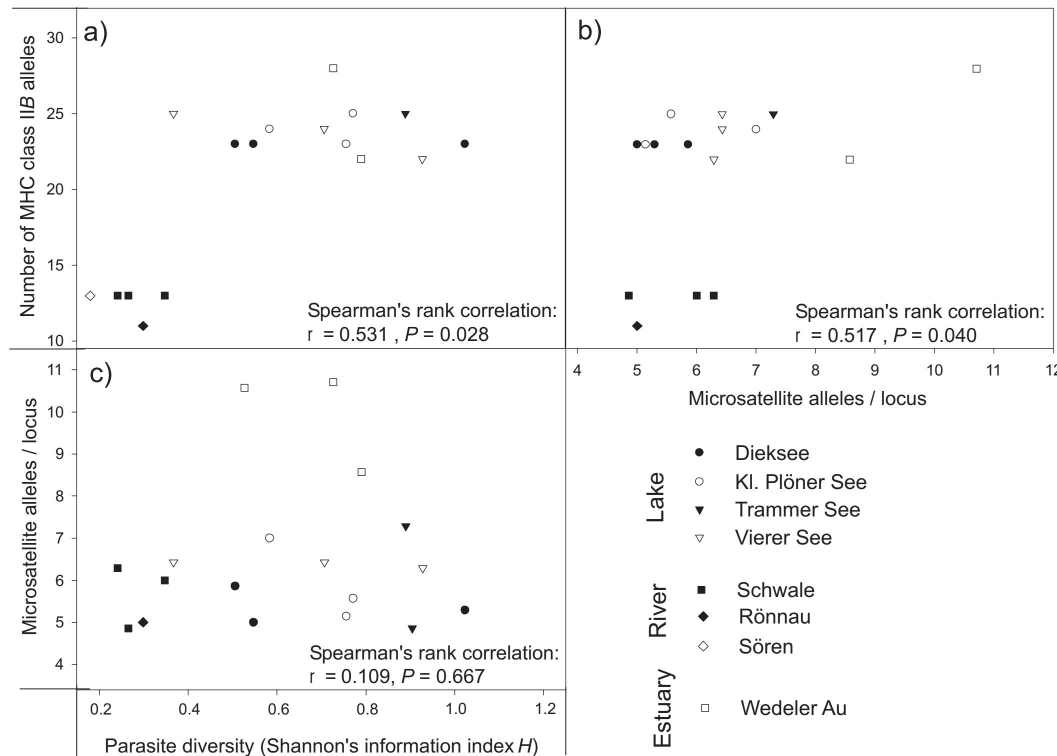


Figure I-1: Populations of Three-spined stickleback (*Gasterosteus aculeatus*): Relationships between neutral, genomewide variability (measured as microsatellite alleles / locus), parasite diversity (measured as Simpson's diversity *D*) and immunogenetic diversity (measured as number of distinct alleles present within populations). Since we found genetic differentiation between sampling dates at several occasions (Dieksee Sept – Nov: F_{ST} = 0.010*, Kl. Plöner See Sept- Nov: F_{ST} = 0.027***, Vierer See Sept – Mar: F_{ST} = 0.011*, Wedeler Au Sept – Mar: F_{ST} = 0.016** Nov-Mar: F_{ST} = 0.015** with ***: p < 0.001, **: p< 0.01 * < 0.05) each sampling date of all populations were considered separately in the analysis. However, patterns are consistent when dates are pooled (MHC alleles vs. Simpson's D: ρ = 0.745, = 0.009; MHC vs. microsatellite alleles/locus: ρ = 0.780, *P* = 0.009; microsatellite alleles/locus vs. Simpon's D: ρ = -0.409, *P* = 0.169). Panel a) shows the relationship of MHC diversity to parasite diversity, panel b) shows the relationship of MHC to microsatellite alleles / locus and panel c) shows the relationship of parasite diversity to genomewide variability.

However, particular habitats were always associated with relatively high or low MHC diversity, with river sites having fewer alleles than lake habitats (Fig. I-1). At the same time, the river samples showed significantly lower diversity in terms of parasite community and immunogenetics than lake and estuary populations, which were not different from each other. But when comparing lakes with the two other habitat types using microsatellites as evolutionary null-model, there was only a small difference between lakes and rivers (Fig. I-2), while microsatellite allelic richness was highest in the estuarine population. Above population-level differences were still present when pooling time points to single populations, microsatellite alleles/locus: $F_{1,5}$ = 4.636, P =

0.084, number of MHC alleles: $F_{1,5}$ = 223.908, P < 0.001, parasite diversity: $F_{1,5}$ = 8.261, P = 0.035.

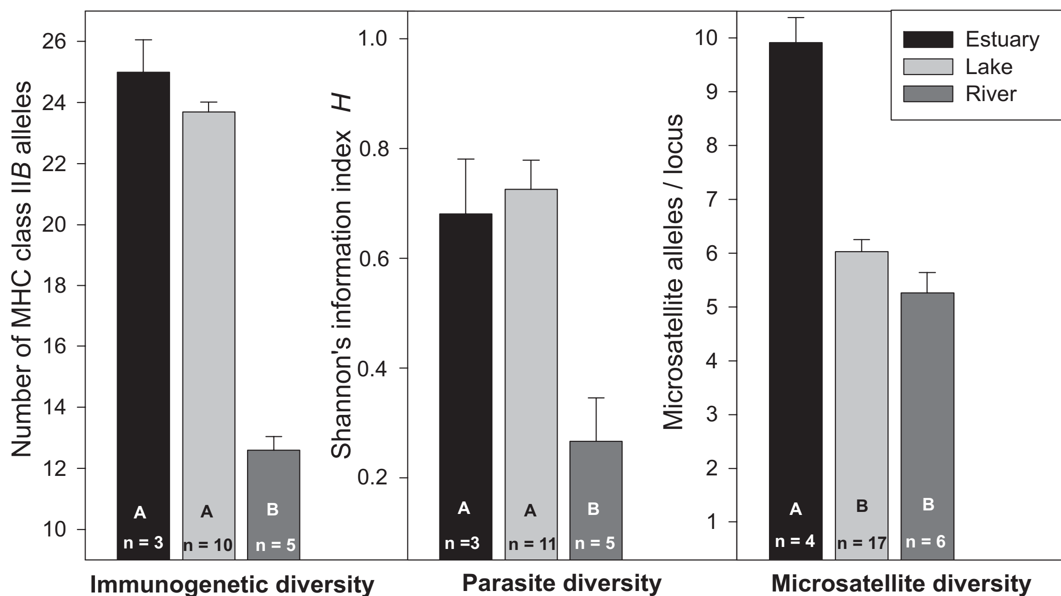


Figure I-2: Three-spined stickleback (*Gasterosteus aculeatus*): Influence of habitat type (lake vs. river vs. estuary) sampled on parasite diversity (measured as Shannon's information index *H*), numbers of MHC class II*B* alleles and microsatellite alleles/locus (± 1 SE). Significant effects of habitat were found for all measurements (least square regressions with sampling date and habitat as factors, number of MHC alleles: *F* ratio 99.454, P < 0.001, parasite diversity: *F* ratio 9.759, P = 0.004, microsatellite alleles/locus: *F* ratio 21.338, P < 0.001). Sampling date did not show a significant effect in the model (*F* ratios ranged from 2.047, P = 0.176 for parasite diversity to 2.66, P = 0.114 for microsatellite alleles/locus). Pairwise differences were calculated using Tukey's Post Hoc test and letter codes (A,B) show group membership.

Parasite load and individual MHC-diversity

We find support for our prediction of an optimal individual MHC diversity when correlating mean residual parasite load to the number of MHC alleles per fish (Fig. I-3). Following this prediction we fitted a quadratic polynomial as the simplest function possessing a minimum and found the lowest parasite load at intermediate 5.17 (4.36 – 6.48 covering 95% of 1000 bootstrap replicates) alleles/fish. This function clearly explained more of the variation in the data than linear (R^2 = 0.151) or increasing functions (exponential rise: R^2 = 0.109). Furthermore individual genome-wide heterozygosity could not explain individual number of MHC alleles (R^2 = 0.004, $F_{1,253}$ = 1.006, P = 0.317) nor parasitation (R^2 < 0.001, $F_{1,253}$ = 0.352, P = 0.554), thereby excluding general heterozygote advantage. The polynomial regression remains significant when jack-knifing over allele numbers (Fig. I-3). To our knowledge this is

the first empirical evidence for Nowak et al.’s (Nowak et al. 1992) theoretical prediction for an optimal number of MHC alleles in individuals.

Discussion

Parasite versus MHC-diversity in populations

The extensive polymorphism in the 8 studied populations (13 to 28 different MHC class II*B* alleles, Tab. I-1) indicates balancing selection working on the MHC loci. However, genetic drift which depends on parameters such as effective population size (N_e) also seems to have an influence on the amount of variability within the MHC. Accordingly, we found a positive correlation of microsatellite allele richness with MHC variability (Fig. I-1b), which is in line with other recent findings (Gutierrez-Espeleta et al. 2001; Hedrick et al. 2001b). Nevertheless, if parasites are the selective force behind the maintenance of MHC polymorphism parasite diversity should rather correlate with MHC variability than with genome-wide variability. Our data support this notion as populations being exposed to a wider array of macroparasites were also more diverse in terms of their MHC class II*B* genes (Fig. I-1a). The relative strength of the partial correlation outweighs that of MHC to microsatellite alleles/locus (see Fig. I-1a,b). On the other hand, we find no correlation of parasite diversity with genome-wide variability (Fig. I-1c). Accepting that MHC allele frequencies are not solely governed by neutral processes, but are also subject to parasite-induced selection, a low parasite diversity should be equivalent to a relaxation of selection (Van Valen 1965) making genetic variability at resistance genes less important for survival of the population.

Unfortunately, parasite diversity is confounded with two major habitat types sampled in this study, lakes and rivers. The relationship among MHC and parasite diversity is weak within single habitats, while the data rather cluster according to habitat (Fig. I-1a). In a previous study (Reusch et al. 2001b) we have already identified a phylogeographic signal indicating virtually no gene flow between even physically adjacent populations from different habitats. Similar rapid ecological speciation has been reported from species pairs on Vancouver Island, which exploit different food niches (Schluter 1996). We cannot rule out other such confounding effects, albeit any ecological speciation depending on trophic niche is probably irrelevant for immunogenetic variation. One possible diverging process operating between habitat types may be different demographic histories or different effective population sizes N_e. Our and previous

(Reusch et al. 2001b) microsatellite data as well as general expectations from other fish species (DeWoody and Avise 2000) suggest that N_e of the estuarine population is largest, whereas there is no significant difference between lakes and rivers based on our findings (Fig. I-2). Therefore, the effect of genetic drift seems to be comparable in these two habitats. Variability at the MHC loci could be expected to show the same pattern, but instead we find elevated levels of immunogenetic diversity in lake populations relative to river populations. Such a pattern can more easily be explained by selective impact of parasite communities, which show the same pattern between habitats as MHC (Fig. I-2). In conclusion, we have found supportive evidence for our initial hypothesis, but more phylogenetically independent populations need to be assessed to formally test the hypothesis and clarify the confounding effects induced by differences in habitat .

Parasite load and individual MHC-diversity

If individuals with more MHC-alleles are able to present more foreign peptides to T-cells we expected that immune responses could be mounted to resist a wider array of parasites. Additionally, following the theoretical prediction of an optimal number of MHC alleles within an individual (Nowak et al. 1992) we found higher parasite burdens at both extreme ends of the distribution (Fig. I-3). We fitted a quadratic polynomial as the simplest function possessing a minimum on pooled data from all fish as the optimality function should be an intrinsic feature of the stickleback MHC. We found the lowest parasite load at 5.17 (4.36 – 6.48 covering 95% of 1000 bootstrap replicates) alleles/fish. The rise in parasite burden may indicate prior elimination of self-reactive T-cell lines in those individuals. This may only be important in populations with relatively high individual parasitic burden, e.g. lakes. In rivers, on the other hand, we could not find this pattern. Here, fish are frequently challenged with infections from only one parasite species (Kalbe et al. 2002). The chance of mounting a functional immune response against a single pathogen does not depend on high levels of T cell diversity. Rather effective presentation of the antigen by a MHC allele and recognition by a single T cell receptor line is necessary. Such an effective modulation can be achieved by a single specific MHC allele. Even though the optimal number is an intrinsic property of MHC-T cell complexes and should be global for sticklebacks, self elimination effects only appear in fish suffering from infestation by a wider array of parasite species. Note that the distribution of parasite load on number of alleles is right-skewed which may indicate combinatoric interaction between the two antigen-presenting parts of the

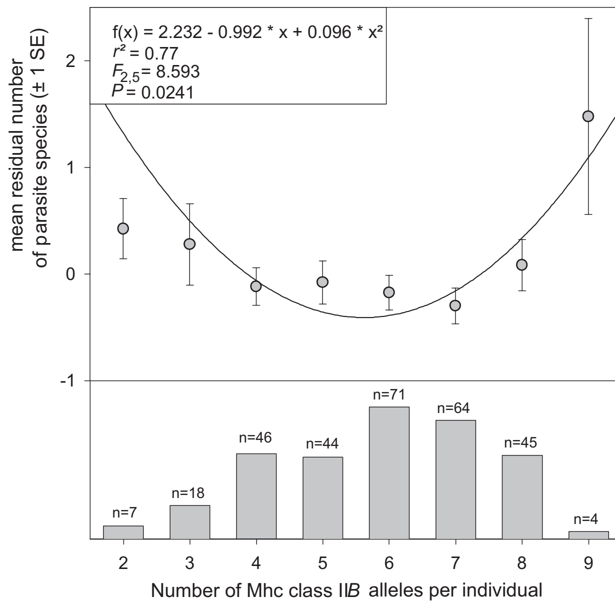


Figure I-3: Three-spined stickleback (*Gasterosteus aculeatus*) from 8 populations in Schleswig-Holstein: Relationship of residual parasite load (± 1 SE) and number of MHC class IIB alleles present in individual fish. Parasite diversity varies markedly between populations (see also Kalbe et al., 2002) around a global average of 3.86 parasite species / fish. To reduce variation introduced by effects of population affiliation residuals of an ANOVA with population as factor (ANOVA $F_{5,251}$ = 38.06, P < 0.001) were used for subsequent analysis. The fitted quadratic polynomial has a minimum of 5.17 (95% C.I.: 4.36 – 6.48, 1000 bootstrap replicates over individuals). Jack-knifing over allele-numbers resulted in six significant (P < 0.05) quadratic polynomials with minima lying within the range of the bootstrap replicates. The distribution of allele numbers in the data set is shown in the histogram below.

heterodimer (extracellular domains of α and β chain (Mason 2001)). This might also explain the rare occurrence of fish bearing maximal diversity with nine alleles as these fish will be strongly limited in their functional repertoire of T-lymphocytes (Fig. I-3).

Recent findings of Reusch et al.(2001a) and Aeschlimann et al. (2003) suggest that 'allele counting', a strategy of sexual selection, is also influencing MHC-diversity in offspring. Female sticklebacks with low individual diversity prefer the odor of males with more MHC alleles, while females with high individual diversity prefer males with low number of MHC alleles. Females ultimately aim for a total of 10 different alleles combined from their own and from the male's. Our findings identify the adaptive value of such a sophisticated choice mechanism. By aiming at 10 different alleles (male + female) the fitness of progeny will be maximized as five alleles on average can be expected in the offspring after recombination, which is strikingly similar to the number of alleles with minimal parasite load from our data (i.e. 5.17).

As fish with an optimal level of diversity suffer least from parasitation, overdominance in this multi-locus system can explain the extraordinary polymorphism of stickleback MHC class II*B* genes. The effects of multi-locus overdominance and the pattern found between habitats only appear when considering many naturally occurring parasite

species simultaneously. Of the 434 examined sticklebacks none was parasite free and only one specimen harboured a single parasite species (Kalbe et al. 2002). On average, 3.86 different parasite species were found per fish. It cannot be expected that sticklebacks suffer from an exceptionally high parasite burden compared with other host species given the sheer amount of parasites among all biota (Windsor 1998). Many studies investigated only the effect of a single parasite or pathogen (Hill et al. 1991; Thursz et al. 1995; Paterson et al. 1998; Godot et al. 2000; Langefors et al. 2001) and identified associations between infections and certain MHC alleles. Such associations are prerequisite for balancing selection, but can only cause the striking diversification as combined effects from multiple parasite-host interactions.

Conclusions

In this paper we found a consistent relationship between parasite diversity among different habitats and MHC diversity. Although selectively neutral processes contributed to MHC correlated genetic structure, parasite diversity explained significantly more variance.

At the level of individuals, overdominance is the mechanism behind the balancing selection observed. Here, we understand overdominance as general diversity advantage, whereas the classical narrow sense definition is restricted to the synergistic interaction between two alleles of the same locus (Parsons and Bodmer 1961). In the case of duplicated MHC loci, polymorphism can be maintained by a fitness advantage of individuals possessing different gene products at multiple, functional equivalent loci, even if they are homozygous at the individual gene locus. Furthermore, individuals cannot achieve an optimal MHC-class II*B* diversity in terms of parasite resistance with a single-copy gene (Fig. I-3).

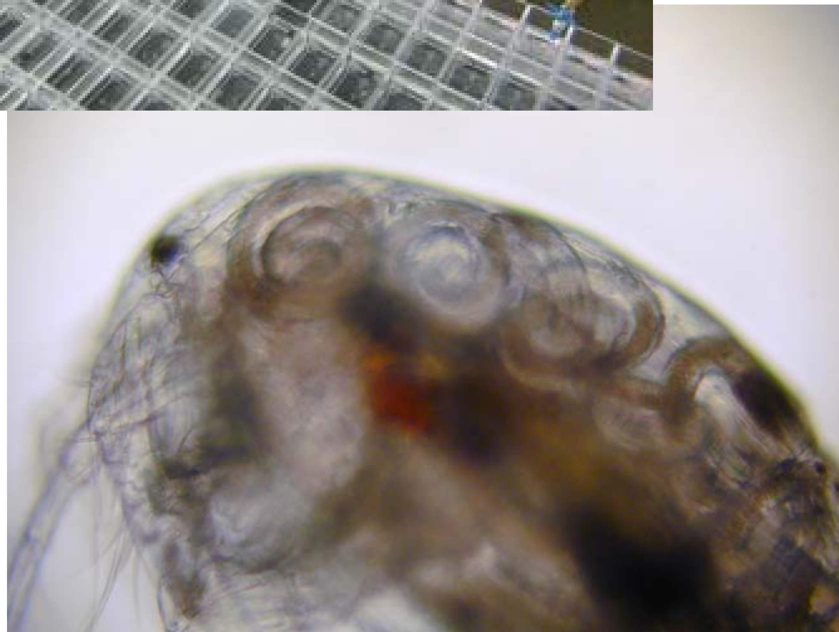
Individual parasite load cannot be reliably assessed by the choosing sex. As a solution for choosiness, Hamilton & Zuk (1982) interpreted sexually selected ornaments as honest signals for immunocompetence. In our data set the relationship between number of MHC alleles and parasite load suggests that immunogenetic variability can be used as a direct, reliable cue. This does not exclude that other sexually selected traits (e.g. nuptial colour, Milinski and Bakker 1990) are advertising other resistance traits. As not only the number of alleles matters, but also their identity, nuptial colour might be an additional cue for immunocompetence (Milinski and Bakker 1990). We also suggest that in this coevolutionary situation, the identity of beneficial alleles will vary both

spatially and temporarily, precluding a fixation of individual haplotypes for an optimal level of diversity (i.e. 5 variants of MHC class II*B*).

Surprisingly, in the case of MHC correlated sexual selection, individual MHC diversity will not become exaggerated as a handicap or run-away selection process but is instead optimised towards minimal parasite load (Aeschlimann et al. 2003). This shows that parasites can be regarded as a potential cause for natural and sexual selection. Both mechanisms of selection would interact to result in the striking MHC polymorphism found in the wild.

Experimental infections

Photo: M. Kalbe



Copepod infected with nematode larvae

Photo: M. Kalbe

CHAPTER II

Parasite selection for immunogenetic optimality

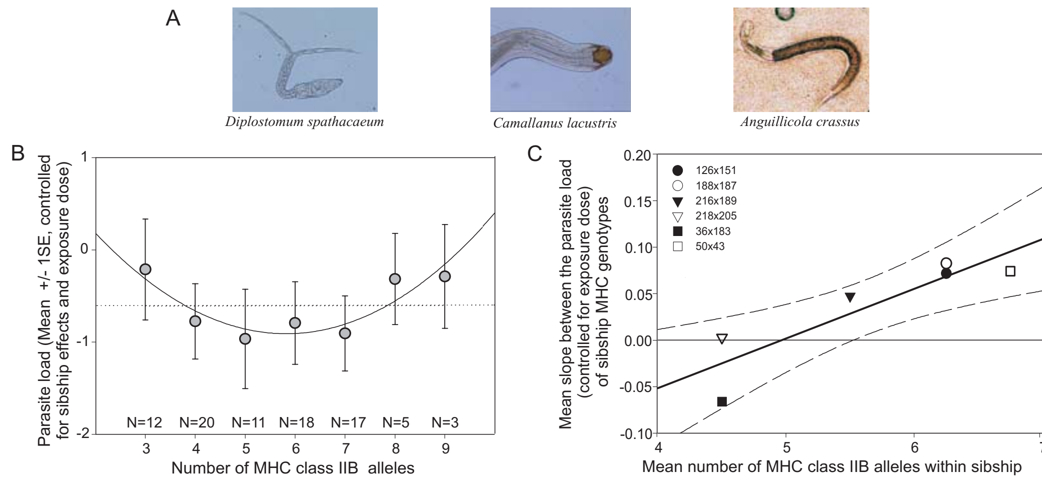


Figure II-1: A) Parasite species used to infect three spined stickleback (*Gasterosteus aculeatus*) B) Relationship between number of expressed MHC class IIB molecules and mean parasite load (expressed as summed residuals from GLM analysis, models included exposure dose as covariate and sibship as random factor, *9*) for double exposed fish. The function matches a quadratic polynomial (r^2 = 0.79, ANOVA $F_{2,4}$ = 7.38, p = 0.045) with a minimum of 5.82 alleles only when considering the combined effect from all three species (fit of polynomials for residuals from single parasite analysis: *A. crassus*: r^2 = 0.06, $F_{2,4}$ = 0.12, p = 0.886; *C. lacustris*: r^2 = 0.48, $F_{2,4}$.= 1.86, p = 0.268; *D. spathaceum*: r^2 = 0.01, $F_{2,4}$ = 0.80, p = 0.991). The dotted line shows the mean from all residuals. C) Relationship of mean number of expressed MHC class IIB molecules per sibship and the mean of the slopes between parasite loads (controlled for exposure dose) associated with the four MHC class IIB genotypes of the corresponding sibship (*9*). The linear relationship (f(x) = -0.265 + 0.053x, r ² = 0.79, $F_{1,4}$ = 15.04, P = 0.013) has a zero intercept at 4.96 alleles, which can be equated with the immunogenetic optimum. Broken lines show 95% C.I..

In vertebrates, genes of the major histocompatibility complex (MHC) with their pronounced polymorphism potentially represent outstanding examples for the selective advantages of genetic diversity (Bernatchez and Landry 2003). Theoretical models predicted that, within an individual, MHC genes can be subjected to two opposing selective forces resulting in an optimal level at intermediate individual MHC diversity (Nowak et al. 1992; Borghans and De Boer 2000, Borghans et al. 2003). Diversifying selection increases heterozygosity and enables wider recognition of pathogens (Penn et al. 2002). This process is opposed by the need to delete T-cells from the repertoire, which react with self peptide – MHC combinations (Vidovic and Matzinger 1988),

which has been proposed as a possible mechanism constraining expansion of MHC genes. Since too high MHC diversity might delimit T cell diversity, it might also impose limitations on the efficiency of pathogen recognition. However, empirical evidence demonstrating fitness benefits in terms of parasite resistance caused by this type of optimal MHC diversity has been lacking. We, therefore tested whether, or not, three-spined sticklebacks (*Gasterosteus aculeatus* L.) carrying an intermediate level of individual MHC diversity, also displayed the strongest level of resistance against parasite infection. Sticklebacks are particularly suited to test MHC optimality, because MHC class II genotypes can differ markedly in the number of MHC class IIB alleles (Reusch et al. 2001a). We caught fish from an outbred population and used these to breed six sibships of immunologically naïve fish (i.e. no previous contact to parasites). Immunogenetic diversity ranged from three to nine MHC class IIB alleles found in reverse transcribed mRNA (see Reusch et al. (2001a) for details on genotyping). The MHC genotypes within these sibships segregated above and below the hypothesized optimal number of ≈ 5 MHC class IIB alleles, which had previously been estimated in an epidemiological field survey (Wegner et al. 2003b, Chapter I).

In individual infection treatments, fish from all sibships were simultaneously exposed to three of the most abundant parasite species that have been identified in the field (Fig. II-1A; (Kalbe et al. 2002). After two rounds of infection, separated by an interval of eight weeks, we found a significant minimal mean infection rate at an intermediate number of individual MHC class IIB variants (i.e. 5.82 expressed alleles, Fig. II-1B). This result was also confirmed when sibships were considered separately (i.e. 4.96 alleles, Fig. II-1C). The strong pattern only appeared when infection with all three parasites was accounted for simultaneously. This may not be not surprising, since single alleles are expected to correlate with single diseases and multiple alleles can contribute to resistance against several infectious agents (Penn et al. 2002).

Infection decreased host body condition, which is a fitness relevant trait in sticklebacks (Wootton 1984). Thus, intensity of infection correlated negatively to the change in body condition of the fish during the experiment (repeated measures ANOVA including control and singly infected fish, total number of parasites * time $F_{1,141} = 4.351$, $p = 0.039$). Therefore, we have provided experimental evidence that multiple parasites can select for optimal, rather than maximal MHC diversity, and that intermediate rather than maximal genetic diversity confers the highest fitness.

Material and methods

Stickleback sibship selection

Previously MHC-genotyped (for methods see Binz et al. (2001; Reusch et al. 2001a) sticklebacks from the Großer Plöner See, Northern Germany, were systematically bred in order to obtain highest possible degrees of individual immunogenetic diversity (i.e. number of MHC class IIB alleles) in offspring. After spawning eggs were removed and 16 eggs of each clutch were used for genotyping and thereby singling out informative sibships. Genomic DNA was extracted with DNeasy Tissue Kit (Qiagen, Hilden) and MHC class IIB sequences were amplified with 2 PCR reactions covering ~80% of present alleles (Binz et al. 2001; Reusch et al. 2001a). We found six informative sibships comprising genotypes with variation around the a priori determined optimum of 5 different sequences. Numbers of MHC class IIB sequences ranged from 3 to 9. Details of sibships can be found in Tab. II-1.
The remaining eggs of these clutches were kept in single, aeriated glass jars until hatching. Fry was split into groups of 80 (later 30) fish to avoid crowding.

Cultivation of parasites

The two nematode species *Camallanus lacustris* and *Anguillicola crassus* have shown to be harmful in other fish species. Especially, the recently introduced *Anguillicola crassus* causes severe damage to European eel populations (Barus and Prokes 1996; Ashworth and Kennedy 1999; Wurtz and Taraschewski 2000), and we found thickened swim bladders and tissue liaison in several cases during dissection of sticklebacks (personal observation). *C. lacustris* is a blood feeder and therefore also causes tissue damage(Moravec 1994). Both parasites are trophically transmitted by copepods. Therefore, we infected lab bred *Macrocyclops albidus* with L1 larvae obtained from swim bladders of adult European eel (*Anguilla anguilla*) in the case of *A. crassus* and L1 larvae from guts of adult perch (*Perca fluvitalis*) in the case of *C. lacustris*, respectively. Both final hosts were supplied by local fishermen and caught in local lakes connected to the Großer Plöner See (i.e. Dieksee). Copepods were kept in single wells of 24-welled cell culture dishes for at least 20 days to guarantee maturation of the larvae. Shortly before infection of the fish the infection status of the copepods was determined by counting nematode larvae inside the copepod with best possible accuracy. Number of larvae within copepods ranged from 1 to 12 for *A. crassus* and 1 to

8 for *C. lacustris*. Accurate counting was sometimes difficult when more than six larvae ended up in an relatively opaque copepod. Especially infection of *A. crassus* larvae was very effective making counting impossible in some cases. For later analysis the number of larvae in these cases was substituted by the mean of all copepods to which a number >6 could be assigned with certainty, i.e. 8.32.

The eye fluke (*Diplostomum pseudospathaceum*) is harmful to fish as it impedes host vision. Infestation by only a few metacercariae imposes severe limitations on prey detection (Owen et al. 1993) and flight capabilities (Crowden and Broom 1980). Cercaria are released by the first intermediate host, the freshwater snail *Lymnaea stagnalis*, and reach the eye of the fish by active penetration. Snails were collected in a local lake and infection status was determined by exposing single snails to a water change under a direct light source which induces the release of cercariae. On the day of infection five snails were randomly chosen out of the pool of infected snails and isolated for 90 minutes and cerceriae from all snails were then pooled according to their density in order to achieve similar contributions.

Infection

Thirty fish were randomly chosen from the six sibships for infection. Out of these, 5 fish each were assigned as control (no exposure), 1st exposure and 2nd exposure, with the remaining 15 fish being exposed twice. Due to small clutch size in sibship 216x189, which comprised only 17 fish left for infection, we decided to expose all fish of this sibship twice and leave out controls. During infection fish were kept in single 3 l aquaria and starved for two days before infection. Before each round of infection fish were weighed and length was taken from snout to the tip of the tail. In the first round of infection two copepods carrying *A. crassus* larvae were fed to each fish resulting in an average infection dose of 10.21 (±0.07 s.e.). On the following day 1-2 copepods carrying *C. lacustris* larvae were fed (mean infection dose 2.83 (±0.14 s.e.)) and on day three 20 *D. pseudospathaceum* cercariae for each fish to be infected were transferred out of the cercariae mix (see above) into single petri dishes, which were in turn placed in to fish aquaria simultaneously to eliminate age effects of the cercariae.

One day after *D. pseudospathaceum* exposure fish were put into single flow-through aquaria (16 l) until second exposure, 8 weeks later. Before the second round of exposure additional to measuring we checked for infection by *D. pseudospathaceum* from exposure 1 by counting metacercariae in the eye lenses under a dissection microscope.

After that, infection procedure from round one was repeated in a similar fashion, except for feeding always two *C. lacustris* copepods. In exposure 2 infection doses were 9.93 (±0.05 s.e.) for *A. crassus*, 6.08 (±0.02 s.e.) for *C. lacustris* and 40 cercariae for *D. pseudospathaceum*, respectively.

Dissection and genotyping

In the third week after exposure 2 all fish were subjected to dissection. We dissected fish from one sibship per day (i.e. 30). These were killed and blood samples were taken. Then the swim bladder was extracted and prepared on a microscope slide. Parasites were counted in the swim bladder (*A. crassus*), gut (*C. lacustris*) and both eye lenses (*D. pseudospathaceum*). We could discriminate between infection from exposure 1 and 2 in the case of *D. pseudospathaceum* because we counted metacercariae in the eye lenses after exposure 1 in vivo and for *C. lacustris* as these worms grow continuously within the host resulting in two easily differentiable size classes. *A. crassus*, on the other hand did not show distinct size classes but rather a continuous range, leaving the origin of the single worm unknown. Organs (liver, head kidney and spleen) were collected for further analysis. Table 1 shows the details of infection intensities. Spleen was transferred to RNAlater solution (Qiagen) for later RNA extraction using RNeasy spin columns (Qiagen) including on column DNA digestion with DNAse I (Qiagen). Messenger RNA was reverse transcribed to cDNA using Omniscript RT (Qiagen) and anchored oligo-dT primers (WT_{24}). Fish were then genotyped as described for the eggs above, only on the functional level of cDNA.

Data analysis

The distribution of macroparasites within hosts can be approximated with a negative binomial distribution (Shaw et al. 1998). Consequently, we fitted negative binomial distributions for total numbers of parasites found at dissection and compared these with other distributions (e.g. normal, poisson). The observed patterns could most adequately be described with negative binomial distributions (summed weighed residuals: -0.08 for *D. spathaceum*, 0.22 for A. crassus and 0.34 for *C. lacustris*, respectively). It can be expected that an MHC dependent effect should be stronger following an adaptive immune response on infections resulting from exposure 2. However, as we could not

differentiate between sources of infection in the case of *A. crassus*, we decided to use total numbers of all parasites rather than excluding all information from one species.

Table II-1: Characteristics of the six stickleback sibships used in this study: the four MHC class IIB genotypes are listed including *n* per genotype and allelic composition. S denotes alleles amplified by the standard primer pair and M alleles amplified by the modified primer pair. Parasitation data includes the sum of infection rates of the three parasite species and counts of the single species (± s.e.).

Sibship (n = number of fish successfully genotyped)	Number of MHC class IIB molecules per genotype (n per genotype)	Alleles in genotypes (S: Standard, M: Modified)	Sum of infection rates	*Camallanus lacustris* (Mean number ± s.e).	*Anguillicola crassus* (Mean number ± s.e).	*Diplostomum pseudospathaceum* (Mean number ± s.e).
126x151 (n=27)	4 (10)	S89,S92 M89b,M93	1.02.	2.57 ± 0.81	2.86 ±2.05	2.85. ± 0.46
	6 (5)	S89,S92 M89b,M89c,M91b,M93	0.61	2 ± 1	1 ±1	3 ± 1
	7 (5)	S89,S90,S91b M89c,M90,M91b,M92	0.92	2 ± 1	2 ±2	3 ± 0
	8 (7)	S89,S90,S91b,S92 M89b,M90,M92,M93	1.05	5 ± 1.58	1.75 ±0.75	2 ± 0.71
188x187 (n=29)	4 (5)	S90,S91 M90,M92	1.17	3.5 ±3.5	0 ±0	7.5 ± 0.5
	6 (8)	S89,S89b,S92 M89b,M92/93,M93	1.01	2.25 ± 0.85	1.5 ±1	9.5
	7 (11)	S89,S90,S91 M89b,M90,M92,M92/93	0.96	2.22 ± 0.54	0.67 ±0.29	8.33 ± 2.02
	8 (5)	S89, S90, S91,S92 M89b,M90,M92,M93	1.40	2 ±0.54	1 ±0.33	5 ± 1.37
216x189 (n=16)	4 (4)	S89 M89c,M90,M91b	0.58	1.25 ± 0.75	1.5 ±0.5	4.5 ± 0.65
	5 (7)	S89,S91b,S93 M89b,M92/93	0.42	1.29 ± 0.56	0.71 ±0.36	3 ± 0.65
	6 (1)	S89,S91b,S93 M89c,M90,M91b	1.51	4	2	3
	7 (4)	S89,S91b,S93 M89b,M89c,M90,M92/93	0.44	0.75 ± 0.25	0.5 ±0.5	2.25 ± 0.95
218x205 (n=27)	3 (9)	S89 M89,M89b	0.79	2.57 ± 0.75	1.14 ± 0.14	3.57 ± 0.57
	4 (6)	S89,S92 M89b,M92/ 93	0.1	0 ± 0	0 ±0	3 ± 2
	5 (5)	S89,S92 M89b,M91b,M92/93	0.42	0.5 ± 0.5	2.5 ±2.5	1 ± 0
	6 (7)	S89,S92,S93 M89b,M91b,M92/93	0.71	3 ± 2	0 ±0	3 ± 2
36x183 (n=29)	3 (10)	S90 M89c,M90	1.00	2 ± 0.45	2.2 ±0.73	4.6 ± 1.44
	4 (9)	S89,S90 M89,M90	1.12	4 ± 1.10	0.6 ±0.4	3.8 ± 0.86
	5 (2)	S90,S91b M89c,M90,M91	1.29	3	1	8
	6 (8)	S89,S90,S91b M89,M90,M91	0.73	2.5 ± 1.19	0.25 ±0.25	4.25 ± 1.44
50x43 (n=25)	5 (3)	S89,S91,S93 M89c,M91b	0.50	2 ± 0.66	1 ±0.4	4 ± 0.6
	6 (8)	S89,S92,S93 M89b,M91b,M92/93	0.83	2.2 ± 0.88	0.6 ±0.58	4.6 ± 1.52
	7 (10)	S89,S90,S91 M89c,M90,M91,M91b	0.51	1.67 ± 1.15	1	4
	9 (4)	S89,S90,S91,S92,S93 M89b,M90,M91,M92/93	1.00	3 ± 2.2	1 ± 1	6 ± 2.65

Parasite resistance is likely to be controlled by several genes besides MHC genes. As we did not control for effects of these genes, more closely related individuals from the same sibships will differ systematically from those of other sibships in infection rates. Furthermore, due to high mortality in infected copepods we were not able to expose

every fish with the same number of infective nematode larvae. To eliminate variation caused by systematic differences in these factors we used residuals from GLMs for cumulative analysis including all parasites. Separate analysis were used for each species using GenStat 5th edition, release 4.21 (Lawes Agricultural trust). We entered aggregation factors k as obtained from the best fitting distribution (see above) and used sibshib as a random factor and number of infective larvae from all copepods as a covariate, where appropiate. The general influence of immunogenetic diversity (i.e. the number of MHC class IIB alleles) on the sums of these residuals was tested, following our initial hypothesis, by fitting a quadratic polynomial through the mean of the summed residuals per number of MHC class IIB alleles. Fitting this rather unusual function is justified as it comprises the simplest function possessing a minimum. Furthermore the amount of variance explained was compared to other functions (linear, exponential) to exclude more simple functions as better predictors.

Albeit using residuals over sibships, the range of alleles entering the analysis (i.e. 3-9) habours an inter-sibship component as one sibship can only add to four degrees of diversity rather than 7. On the other hand, it is virtually impossible to fit a significant quadratic polynomial through only four points, especially when considering that error becomes larger with only few individuals entering a single class of allele numbers. Therefore, we tried to reduce the information from the three mean infection rates of one sibship into a single data point in order to compare a general relationship valid within all sibships. We calculated slopes of summed infection rates between each pair of allele numbers for each sibship. Assuming that the underlying function really possesses a minimum that is identical for all sibships we would then expect a mean of the 8 slopes within one sibship equals 0 when mean number of alleles within this sibship equals exactly this minimum. Furthermore, the mean of the slopes should be positive when the mean of allele numbers is greater than the minimum and vice versa. Concluding, one can hypothesize that all single sibship data points should fall on one straight line with the x-axis intercept at the hypothesized minimum. Due to unknown properties of this statistic we validated our data by comparing it to 1000 permutated data sets, in which we permutated the observed infection rates over existing sibship-genotype combinations.

Real Time PCR system

CHAPTER III

MHC Class II expression depends on genetic background and individual MHC diversity in three-spined sticklebacks

Abstract

Genes of the major histocompatibility complex (MHC) have been studied for several decades because of their pronounced allelic polymorphism. Structural allelic polymorphism is, however, not the only source of variability subjected to natural selection. Another source might be heritable differences in gene expression patterns. Here, we show that besides considerable allelic variation for MHC class IIB genes of three-spined sticklebacks *Gasterosteus aculeatus* there are strong differences in background MHC class IIB expression patterns. These differences were consistent over two generations indicating a genetic component. Furthermore, allelic diversity within families was negatively correlated to expression level suggesting compensatory upregulation in fish with low allelic, i.e. suboptimal, diversity. Exposure to parasitic infection also upregulated MHC expression within each family. Therefore, the observed differences between families and the negative correlation to allelic diversity might have evolutionary implications for the onset and control of the immune response.

Introduction

Genes of the major histocompatibility (MHC) complex have attracted interest from researchers for several decades - mainly due to their outstanding genetic polymorphism (Klein 1986; Apanius et al. 1997; Bernatchez and Landry 2003). Since the discovery of the extraordinary numbers of MHC alleles in natural populations it was supposed that balancing selection is responsible for their maintenance (Clarke and Kirby 1966). To date in humans, the best studied organism, as many as 563 alleles at the most polymorphic MHC class I locus have been identified (Robinson et al. 2003). Functionally, MHC molecules bridge the gap between self / non-self recognition and the immune system by presenting peptides to T cells. Presented peptides are derived from self and non-self proteins by proteolytic degradation. The two main classes of MHC molecules, class I and class II, fulfil different purposes. Class I genes are expressed on nearly all nucleated cells and present peptides from within the cell to CD8+ cytotoxic T cells.

Class II genes, on the other hand, are only expressed on specialized antigen-presenting cells of the immune system like dentritic cells, macrophages or B cells. Peptides are presented to CD4+ T-helper cells, which initiate the onset of antibody production and immunological memory (i.e. an acquired immune response). An influence of MHC genotypes on the occurrence or course of infection has been demonstrated in several vertebrate taxa (Apanius et al. 1997; Bernatchez and Landry 2003) – some showing significant association between MHC haplotypes or alleles and disease (Briles et al. 1977; Hill et al. 1991; Decamposlima et al. 1993; Godot et al. 2000; Langefors et al. 2001; Grimholt et al. 2003) while others demonstrated the advantages of individual MHC diversity (Carrington et al. 1999; Penn et al. 2002; McClelland et al. 2003; Wegner et al. 2003a, Chapter II; for review see Wegner et al. in press).

Next to allelic polymorphism evidence is accumulating for the evolutionary importance of genetic components in gene transcription (Oleksiak et al. 2002; Morley et al. 2004). In chicken for example, where classical MHC genes are major regulators of disease progression, only one of the two class I genes present in some haplotypes are expressed on the cell surface (Kaufman 2000). In mammals, on the other hand, there are substantial differences in expression levels between alleles (Neefjes and Ploegh 1988), whereas the overall surface presentation of class I gene products averaged over all loci seems to be rather constant (Kaufman 2000). A reason for this might be that purifying

selection acts on the sequence of mammalian class I promoters to guarantee permanent surveillance of intra-cellular pathogens (Mitchison and Roes 2002).

Due to its immediate importance for the onset of the adaptive immune response, modulation of MHC class II expression is generally much more variable over time. Only in case of an acute infection presentation of pathogen-derived peptides becomes highly necessary. Expression is therefore tightly regulated by factors associated with the immune response (Guardiola and Maffei 1993). Modulators and direction of regulation differ between cell types and transcription is additionally influenced by maturation stage of the cell and contact with other cells (Mach et al. 1996). One of the major players regulating MHC class II is interferon γ next to a series of interleukins (e.g. IL-4, -10, -13). The MHC class II promoter binding sites contain four major sequence motifs (called W/S, X, X2 and Y box) approximately located 150 bp upstream of the transcription initiation site (van den Elsen et al. 1998). The order of these motifs was found to be well conserved in the majority of species studied (Glimcher and Kara 1992) suggesting common mechanisms of transcription regulation (van den Elsen et al. 1998). Despite the common structure of these motifs there seems to be considerable polymorphism between single promoter sequences (Mitchison and Roes 2002). Associations between promoter polymorphism and disease have been described for MHC genes (Heldt et al. 2003) and other genes (Humphries et al. 2001) making promoter sequences a direct target for natural selection. Another example for the selective importance of MHC class II regulation is found in polyploid species, which circumvent the negative effects of elevated gene dosage by silencing copies from surplus chromosomes (Du Pasquier et al. 1989; Dixon et al. 1996; vanErp et al. 1996). It has been suggested that polymorphism in promoter regions of MHC class II genes might be selectively maintained to ensure flexibility of mounting the correct type of T cell mediated response (i.e. Th1 or Th2; Mitchison et al. 2000). Classically, allelic polymorphism of the coding sequences of MHC genes attracted more attention (Apanius et al. 1997; Bernatchez and Landry 2003) with heterozygote advantage partly explaining the selective maintenance of genetic variability (Penn et al. 2002; McClelland et al. 2003; Wegner et al. 2004; Wegner et al. in press).

In three-spined sticklebacks *Gasterosteus aculeatus L.* a strong influence of individual MHC class IIB diversity (i.e. the number of different MHC class IIB alleles per individual) on the distribution of parasitic infection was found. Fish with an intermediate number of alleles (i.e. 5-6) suffer least from parasite infection by having

lower levels of oxidative stress and limiting parasite growth (Kurtz et al. 2004) or have lower infection rates in general (Wegner et al. 2003a, Chapter II; Wegner et al. 2003b, Chapter I). Despite of the selective advantage of fish with an optimal number of MHC alleles on the genomic level, little is known about the transcription patterns of MHC class II genes in these fish.

Therefore, we compared the patterns of MHC class IIB expression in six families of sticklebacks previously exposed to three parasite species. If individuals with a lower than optimal individual MHC diversity are put at a permanent disadvantage, because their chances of not having MHC alleles with high affinity against parasitic infection are higher. An upregulation of MHC transcription might theoretically compensate for this. If this is the case, MHC allelic diversity should affect MHC expression levels, thus providing evidence for an additional link between individual MHC diversity and parasitic infection.

We also tested whether there is a functional link between exposure and MHC expression in these fish, hypothesizing that exposure should upregulate MHC expression. Since the constitutive level of MHC gene expression can be blurred by acute infections and might therefore be highly variable, observed transcription levels might be condition dependent. To see whether any observed difference between families harbours a heritable component, we further compared MHC class IIB expression levels between four inbred lines derived from two of the six families. We expected that in the presence of a genetic component, patterns of gene expression are comparable between parental and F1 families, thus providing genetic variation – the handle for natural selection to act on.

Material & Methods

Fish families

To analyse differences in expression of MHC class II genes between different tissues we dissected three individuals from a single family and extracted RNA from spleen, brain, muscle/skin, liver, gills, head kidney and gut. To increase MHC expression (Koppang et al. 1998) these fish had been exposed to 30 *Diplostomum pseudospathacaeum* cercariae. Due to practical reasons we used only one parasite, with which infections are carried out most easily. Starting material was weighed before RNA extraction and immediately transferred into homogenisation buffer.

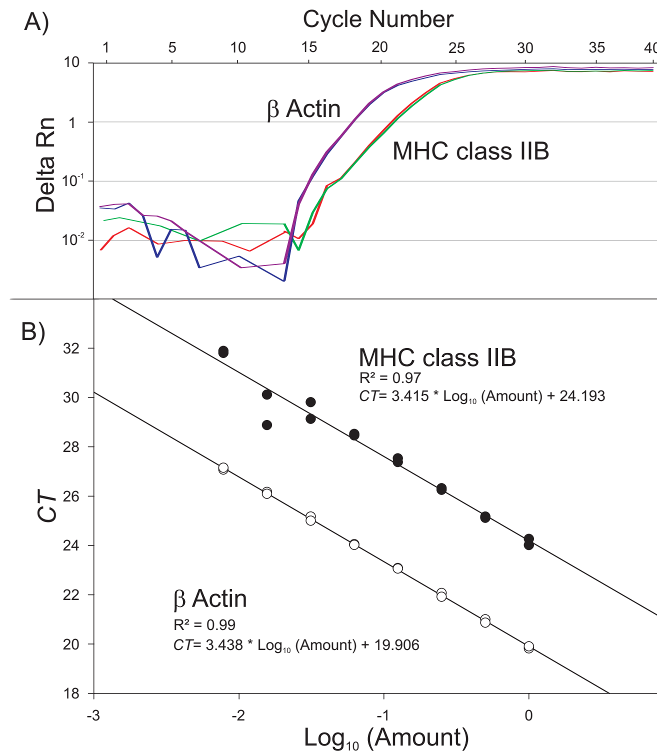


Figure III-1: Panel A): Example real time PCR of β actin and MHC class IIB PCRs in double replicates. Panel B): Dilution series of one cDNA preparation showing the parallel slope of the house-keeping gene β actin and the MHC class IIB product.

For analysis of MHC class IIB expression patterns we extracted RNA from 167 fish stemming from six fish families described in another study (Wegner et al. 2003a, Chapter II). Each family contributed 30 individuals (with the exception of family 216x189, where only 17 individuals were available at the start of the experiment). Out of each of these families five fish were randomly assigned as control while the remaining 25 were either double exposed or single exposed to three of the most common macro-parasites from the population of origin (Kalbe et al. 2002). Details on fish infection protocols can be found in Chapter II. Starting 14 days after the second exposure we dissected one family of fish per day. Fish were weighed, measured and parasite loads were determined. Whole spleens were removed, weighed and stored in RNAlater solution (Qiagen, Hilden) until extraction of RNA.

As we wanted to look for a genetic component of MHC expression patterns we produced inbred lines from four of the six families described above. These families were 188x187, 218x205, 36x183 and 50x43. After determining differences in expression patterns in the parental generation, we chose the two families with the largest difference for analysis of the filial expression pattern. We used two replicate inbred lines per parental family and exposed them to 30 *D. pseudospathacaeum* cercariae analogous to the tissue sample above. Twenty days after exposure we extracted the gill tissue of five fish from each family resulting in 28 individual RNA preparations. Gill tissue was immediately transferred to homogenisation buffer and total RNA was extracted.

RNA extraction and Reverse Transcription

Total RNA was extracted from individual organs (mainly spleen and gill tissue) with the use of the Rneasy Kit (Qiagen, Hilden). Tissue samples were homogenized in tissue lysis buffer containing 1% β-Mercaptoethanol in a Retsch ball mill by shaking samples twice together with a single steel bead for two minutes at a frequency of 20 Hz. During the following preparation of Total RNA residual DNA was removed by on-column digestion with Dnase I provided by the manufacturer (Qiagen, Hilden). RNA was eluted in 30µl RNAse-free water.

Reverse transcription was performed using Omniscript Reverse Transcriptase (RT) kit (Qiagen, Hilden). Single reactions were carried out in volumes of 20µl with 2.0 µl of 10x RT buffer, 0.5 mM of each dNTP, 1µM of anchored oligo-dT primer ($V(T)_{21}$), 10 units of RNase inhibitor and four units of Omniscript Reverse Transcriptase. We used a standardized amount of 1 µg total RNA as template for reverse transcription for the tissue samples to test for constitutive expression of the house keeping gene β-Actin. After proving that β-Actin is constitutively expressed in most of the tissues sampled, we used whole spleens of the 167 experimentally infected fish for RNA extraction. Since spleen size ranged from 0.2 mg to 2.0 mg (mean 0.76 ±0.02 mg) expected average yield for spleen tissue was approximately 0.5-2 µg of template total RNA.

Quality of cDNA and absence of genomic DNA was then confirmed by PCR amplification of a stretch of β-Actin, which covered the boundary from exon 2 to exon 3. Primer sequences were 5'- ATG GAA GAT GAA ATC GCC GC -3' functioning as forward and 5'- TGC CAG ATC TTC TCC ATG TCG-3' as reverse primer. PCRs consisted of an initial 3'00" denaturation at 94°C, followed by 27 cycles of 0'30" denaturation at 94°C, 0'30" annealing at 60°C and 1'00" of extension at 72°C. The cDNA amplicon had a size of 260 bp, while amplification from genomic DNA would result in a product of ≈100 bp longer.

Template quantification by RealTime PCR

We developed a real time PCR assay for relative quantification of MHC class II transcripts against the constitutive house keeping gene β-Actin. For amplification of β-Actin we used the same primers as described above. A stretch of 105 bp was amplified from the exon 2 of the MHC class IIB genes. Primers were designed to amplify all sequences known, so far (T.B.H. Reusch, unpublished). We used 5'-AAC TCC ACT GAG CTG AAG GAC AT-3' as a forward and 5'-CAG TGA AGC CGA CAW ACT

TCC- 3' as reverse primer. Real time PCR was performed using an ABI 7000 sequence detection system (Applied Biosystems, Darmstadt) and ABgene SYBR Quantitative Master Mix containing the ROX dye as an internal reference. Reactions took place in 20µl volumes and contained 70nM of each primer and 2 µl of diluted cDNA template. We carried out 2-step PCRs which consisted of an initial 15'00" of denaturation and hot start polymerase activation at 95°C followed by 40 cycles of 0'15" denaturation at 95°C and 2'00" annealing/extension at 60°C. Fluorescence was measured after each cycle by SYBR green binding to double stranded DNA (Fig. III-1A). Since SYBR green unspecifically binds to any double stranded DNA molecule contaminations with genomic DNA or primer dimers will also be measured. To exclude these error sources we performed dissociation analysis directly after PCR reactions starting with 60°C. We excluded those samples showing dissociation peaks other than the calculated melting temperature of the respective amplicon.

To determine whether the efficiency of both PCRs are comparable we measured a dilution series ranging from undiluted cDNA to a 1:128 dilution. Fig. III-1B shows that our essay is valid within this dilution span, because the slopes of both PCR assays run in parallel (slope MHC class IIB: 3.415; slope β-Actin: 3.438). Additionally, we calculated efficiencies for single PCR reactions by determining the slope of the product concentration during the exponential phase of the PCR using the LinRegPCR v 7.4 software available from bioinfo@amc.uva.nl (Ramakers et al. 2003). If the amount of target DNA is doubled with each cycle of the PCR, the slope in a log-log plot will equal two. However, most PCR reactions do not perform that good. Average efficiencies in our data set were calculated as 1.765 (± 0.004) for MHC class IIB PCRs and 1.856 (± 0.005) for β-Actin PCRs. These differences in efficiency, however only corrobate our results, when absolute quantification is the aim. Since we were only interested in relative differences between transcripts levels of β-Actin and MHC class IIB genes, we could avoid systematic errors by choosing a constant detection threshold (RN = 0.75 in our case). To further illustrate that the efficiency differences are of no concern to our conclusions we performed additional PCR reactions, in which we compared the transcript levels of the exon 2 product mentioned above with those of an exon 3 product. The monomorphic part of the exon 3 was amplified using 5'-CCT ACG TCA GGC TGC ACT CT-'3 and 5'-GGT GGG AGT GGG TCT GGT A-'3 as primers under identical PCR conditions. This test showed that the PCR efficiency of the exon 3 –PCR did not significantly differ from the efficiency of the β-Actin-PCR ($F_{1,14}$ = 1.53, P =

0.238), while threshold cycle values were virtually identical between exon 2 and exon 3 products ($R^2 = 0.998$). Additionally, we checked for temporal variation between single PCRs by repeating a random subset of 10 samples after six weeks. Replicatibility of results was convincing because the correlation between the ΔCT values obtained from the first and the second run was high ($R^2 = 0.865$). Since all differences are minimal and not significant a relative quantification of starting template is valid by comparing the two genes.

Reactions were performed in double replicates and only those reactions were included in the analysis showing a standard deviation below 0.2 in either the MHC or β-Actin PCRs.

Statistical analysis

All statistical test were performed with JMP Professional 5.0.1 (SAS Institute Inc.). Multifactorial designs were analysed with General Linear Models (GLM) using least square means. Significance of single factors was derived from whole model analysis by means of effect test. Unifactorial models were analysed using analysis of variance. For statistical analysis we used negative ΔCT values because they show a normal distribution. In figures, on the other hand, we displayed positive ΔΔCT for easier visual comprehension.

Results

MHC expression in different tissues

We used a tissue assay covering a range of seven different tissues, which are likely to differ in their transcriptional activity of MHC genes (Koppang et al. 1998). To validate that expression levels of β-Actin are constant in different tissues, template amount should be comparable among all samples. We found constant CT values for β-Actin in brain, gill, gut, head kidney and spleen ranging from 13.69 ± 0.31 in head kidney to 14.63 ± 0.31 in brain tissue. CT values were significantly higher in liver and muscle (liver: 15.98 ± 0.39, muscle: 19.33 ± 0.31, $F_{6,10} = 40.685$, $P < 0.001$). Because these tissues do not fit the assumption of constant house keeping gene expression they were excluded from further analysis. If β-Actin is expressed constitutively in the remaining tissues, expression levels should also be comparable between individuals. Our results

support this as transcriptional activity of β-Actin does not significantly differ between the three individuals ($F_{6,7} = 0.612$, $P = 0.569$).

On the other hand, relative expression of MHC class IIB molecules varies strongly between individuals and tissues (Fig. III-2, whole model: $R^2 = 0.943$, $F_{6,7} = 19.195$, $P < 0.001$, individual: $F_{2,7} = 14.301$, $P = 0.003$, tissue: $F_{6,7} = 19.249$, $P < 0.001$). Highest relative expression is found in gill tissue (ΔCT = -4.50) compared to lowest expression in the head kidney (ΔCT = -7.96). Using the average PCR efficiency of 1.81 the relative differences observed would mean a 7.79-fold increase in MHC class IIB molecules between the two different tissues.

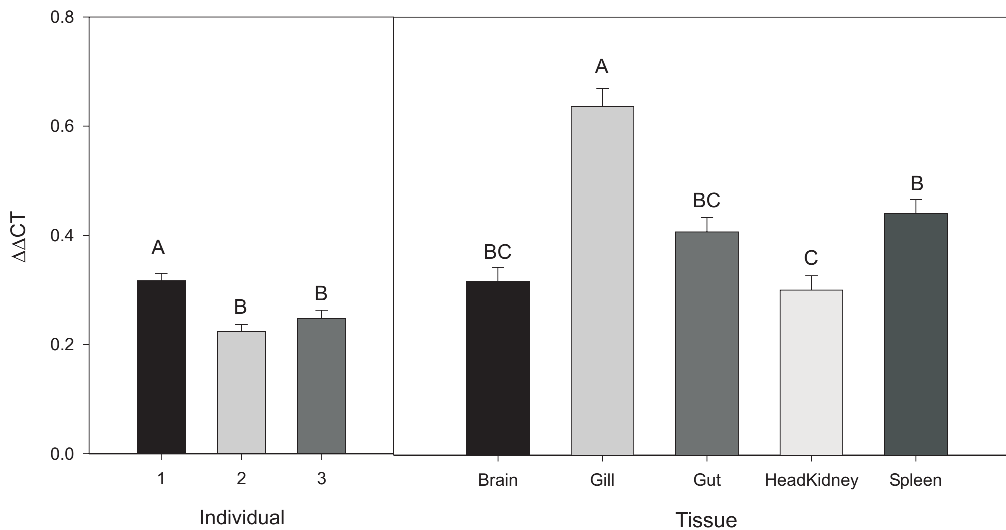


Figure III-2: Mean relative expression of MHC class IIB molecules from a range of tissues. Tissues were extracted from three individual fish. Significant differences were found between individuals (left panel, $F_{2,7} = 14.301$, $P = 0.003$)and between tissues (right panel, $F_{6,7} = 19.249$, $P < 0.001$). Letter codes above the bars indicate significant pairwise differences.

MHC expression in families related to individual MHC diversity

Relative quantification of MHC class IIB expression in spleen tissue of 167 experimentally infected fish (Wegner et al. 2003a, Chapter II) was successful in 115 cases with ΔCT-values ranging from –6.16 to -1.475. We found significant differences between families and an effect of exposure to parasites within families (Fig. III-3, whole model including family and exposure treatment as factors: $R^2 = 0.563$, $F_{6,108} = 23.173$, $P < 0.001$). Exposure to the three parasite species significantly increased MHC class IIB expression by approximately 50% (Fig. III-3b, $F_{1,108} = 4.466$, $P = 0.033$). Even when tested within each single family exposed fish elevated levels of MHC expression

compared to non-exposed control fish in all five families (sign test, P(one-tailed) = 0.031). Interestingly, it did not matter whether fish were exposed once or twice nor how much time elapsed since exposure (mean ΔCT of fish only exposed in first exposure: -3.707 ± 0.276, mean ΔCT of fish only exposed in second exposure: -3.635 ± 0.304, mean ΔCT of fish exposed in both exposure: -3.780 ± 0.138, $F_{2,96}$ = 0.108, P = 0.898). Furthermore, while the exposure dose (i.e. number of infective stages exposed to the host) had a positive effect on MHC expression levels in double exposed fish ($F_{1,57}$ = 5.075, P = 0.028), the actual parasite load (i.e. parasite found during dissection divided by the exposure dose) did not ($F_{1,60}$ = 0.172, P = 0.678).

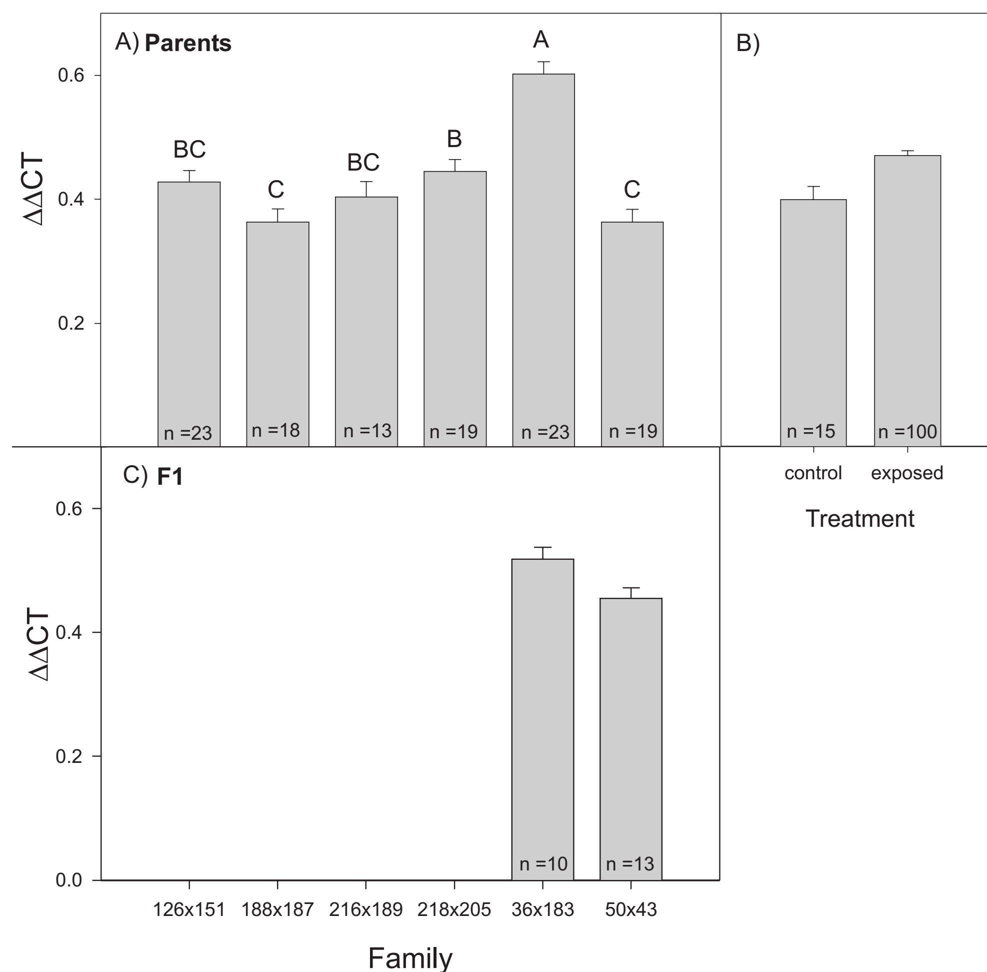


Figure III-3: A) Pattern of relative MHC class IIB expression in six families. Significant differences were found between families ($F_{5,108}$ = 25.793, P < 0.001). B) Expression patterns caused by exposure to three parasite species showing higher levels of MHC class IIB expression in exposed fish ($F_{1,108}$ = 4.466, P = 0.033). C) Pattern of relative MHC class IIB expression in four inbred F1 families derived from the two families showing the most pronounced differences in the parental generation (i.e. 36x183 and 50x43). Relative differences between lines were significant ($F_{1,21}$ = 5.808, P = 0.025) and according to the expected pattern from the parental generation. Letter codes above the bars indicate significant pairwise differences.

Differences among families were much more pronounced (Fig. III-3a, $F_{5,108} = 25.793$, $P < 0.001$) being strongest between family 36x183 (average $\Delta CT = -2.534 \pm 0.189$) and family 50x43 (average $\Delta CT = -4.874 \pm 0.195$). The mean difference of 2.34 cycles would mean a 4-fold increase in MHC class IIB molecules. These families were chosen on behalf of their variation in number of MHC class IIB alleles between the four genotypes within each family. Considering individual fish we found a strong negative correlation between the individual MHC diversity (i.e. the number of MHC class IIB alleles per individual) and the level of MHC class IIB transcription ($R = -0.4194$, $n = 114$, $P < 0.001$). The strong differences between families might, however, confound this finding. A conservative way of testing this relationship is to use family means. Fig. III-4 shows that the negative relationship holds for the six families used ($R^2 = 0.703$, $F_{1,4} = 9.485$, $P = 0.037$).

Since all fish within one family were raised together until first exposure, differences in MHC transcription among families might also reflect the common environment. To test for a genetic component we repeated the expression analysis in four inbred lines derived from full-sibs of the experimental fish, which were all raised in different tanks. From those inbred lines we chose two of the 50x43 and two out of the 36x183 background because differences between these families in MHC expression were highest in the parental generation (Fig. III-3a) and can thus be expected to be highest in the F1, too. We found a significant difference between these lines in the predicted direction, which was, however, smaller than in the parental generation (Fig. III-3c, 0.61 CT difference compared to 2.34, $R^2 = 0.216$, $F_{1,21} = 5.808$, $P = 0.025$).

Discussion

Many studies investigated the parasite fauna of three-spined sticklebacks and the effect of parasitation on stickleback hosts (Chappell 1969; Giles 1983; McPhail and Peacock 1983; Ness and Foster 1999; Poulin 1999; Reimchen and Nosil 2001; Barber and Svensson 2003; Bagamian et al. 2004). Recently, the effect of MHC class IIB genotypes was found to be of prime importance for the outcome of parasitic infection with fish having intermediate individual MHC diversity performing best (Wegner et al. 2003a, Chapter II; Wegner et al. 2003b, Chapter I; Kurtz et al. 2004). This study fills the functional gap between parasite infection and MHC genotypes. Firstly so, because exposure to a mixture of parasites increases MHC transcription levels (Fig. III-3b). Secondly, and more importantly, MHC class IIB expression levels correlate to

individual MHC diversity – in individuals as well as in families (Fig. III-4) showing consistent differences over consecutive generations (Fig. III-3c).

Gene expression of MHC class II is highly variable and expression patterns strongly depend on environmental cues (Guardiola and Maffei 1993). From Fig. III-2 it becomes obvious that MHC class IIB expression is quite variable between individual sticklebacks, too. Messenger RNA levels of MHC class IIB products differed significantly between the three fish used, even though they belonged to the same family and were raised together under identical conditions and treatment. However, when different tissues are compared, a common pattern appears in all three fish. First of all, MHC class IIB transcription is highest in gill tissue (Fig. III-2). Constitutive levels of MHC class II products are also highest in gills of other fish species (e.g. salmon) and levels rise strongly in most tissue types after vaccination (Juul-Madsen et al. 1992; Koppang et al. 1998). Respiratory organs are usually immunologically highly active, because they provide a large interface between the body and the environment. Other immunologically important tissues like the head kidney had rather low expression levels, which can be explained by the fact that a large proportion of cells in stickleback head kidneys are granulocytes (Kurtz et al. 2004; Scharsack et al. 2004), which do not express MHC class II genes.

However, also the composition of cell types within a tissue can change over time as a result of environmental factors. Infection with the tapeworm *Schistocephalus solidus* for example increases the number of granulocytes in sticklebacks (Kurtz et al. 2004; Scharsack et al. 2004). Additionally, the number of granulocytes decreases in the head kidney with increasing individual MHC diversity, while there was no significant effect on lymphocytes (Kurtz et al. 2004). Such a shift in cell populations might cause differences between relative concentrations of MHC class II transcripts between two samples. However, if cell composition correlates between head kidney and spleen, an increase of granulocytes should decrease the concentration of MHC class IIB transcripts. On the other hand, we could show that MHC class IIB expression levels rise due to exposure to parasites in spleen tissue (Fig. III-3b) and expression level drops with increasing individual MHC diversity, which is directly the opposite direction one would expect from the relative number of granulocytes.

Individual MHC diversity plays an important role in the course of infection with the three parasite species. Wegner et al. (2003a) showed that in the same set of fish those with an intermediate number of MHC class IIB alleles have lowest infection levels.

However, MHC expression levels did not directly relate to parasite load in these fish, which is not surprising since the expressed alleles are not alike and confer different degrees of protection against parasitic infection. The quality of the single allele is likely to be more important than the quantity of molecules expressed.

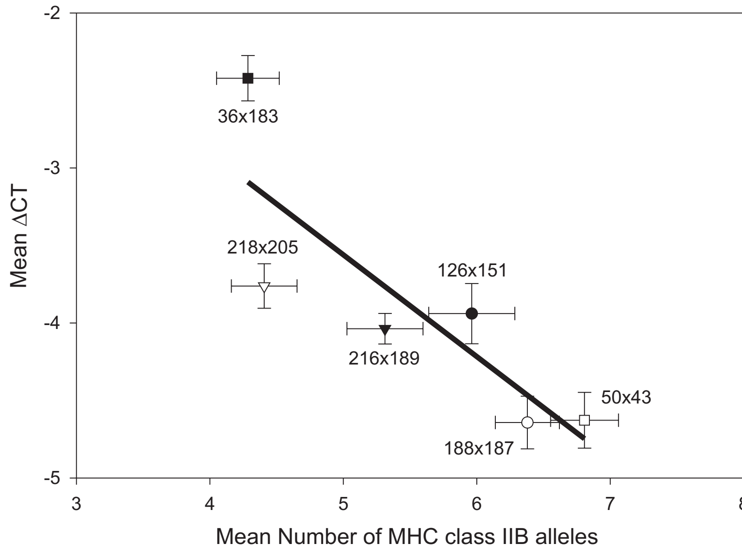


Figure III-4: Relationship between mean number of MHC class IIB alleles within each family (± S.E.) and mean MHC expression level of the corresponding family (ΔCT ± S.E.). The function can be described by a linear regression ($y = -0.657\ x - 0.277$; $F_{1,4} = 9.485$, $P = 0.037$).

Nevertheless, it might make sense for individuals with lower individual MHC diversity to increase MHC expression levels to compensate for lower quality of single alleles. It is less likely for such an individual to possess a MHC allele that confers resistance, i.e. an allele that optimally binds to an immunodominant epitope of the pathogen. However, one of the available MHC alleles might still bind to a certain degree. In such a situation, the lower affinity might be compensated by up-regulating the expression of the low affinity MHC allele and thereby increasing the presentation of less immunodominant peptides on the cell surface. Individuals possessing an optimal number of alleles do not need to increase their general expression level because the high affinity of their alleles for immunodominant pathogen peptides ensures efficient pathogen detection and presentation dosage can therefore be smaller. With regard to parasite resistance, we found an optimum at intermediate individual MHC diversity (Wegner et al. 2003a, Chapter II). Why then did we not find such a pattern for MHC expression? One possible explanation might be that it may not be advantageous for individuals with a number of alleles exceeding the optimal level to increase expression in order to avoid some form of auto-immunity. While there is no direct evidence for such a mechanism the downregulation of expression from surplus copies of MHC class II loci in several polyploid species like *Xenopus* (Du Pasquier et al. 1989) and barbels (Dixon et al. 1996) might hint on a transcriptional mechanism to avoid negative consequences of increased

individual MHC diversity. While gene expression in these cases is nearly switched off, all alleles in high diversity genotypes of sticklebacks are presumably expressed at comparable albeit overall lower levels.

As long as there is no heritable component in expression patterns natural selection cannot act on this trait (Morley et al. 2004). We showed that expression of MHC class IIB genes varies strongly between families (Fig. III-3a). To a certain degree we can exclude environmental factors as a cause for the observed pattern. Fish were, for example, kept singly in randomized order for an extended period of time (10 weeks). Strongest support for a genetic component controlling MHC expression levels in sticklebacks comes from the inbred F1 families (Fig. III-3c), which displayed the same pattern as the parental fish. The difference between families was however smaller than in the parental generation. One reason might be that we used different tissues and expression differences are smaller in gills than in spleens. Furthermore, fish in the F1 were only exposed to one parasite and differences may have been amplified if all pathogens would have been used. Nevertheless, the observed difference indicates a genetic component in the control of MHC class IIB expression, which might be caused by differences in promoter sequences (van den Elsen et al. 1998; Mitchison and Roes 2002). Promoter polymorphism regulating allele specific expression is important for differentiating between Th1 and Th2 type pathways (Mitchison et al. 2000). To guarantee flexibility in mounting the most appropriate immune response, MHC class II promoter polymorphism can be as essential as allelic polymorphism. As discussed earlier, it has to be emphasized that expression levels correlate negatively with individual MHC class IIB allelic diversity. This does not comprise a mutually exclusive explanation since promoter differences might be linked to specific alleles (Mitchison and Roes 2002). However, because alleles are shared between genotypes with different numbers of MHC class IIB alleles within as well as between families, it seems unlikely that allele specific expression is responsible for the observed pattern. Rather the resemblance of MHC allelic diversity between parental and F1 families might cause the similar patterns observed between generations and that heretability of the trait is governed by the number of MHC class IIB alleles.

By establishing a relationship between individual MHC diversity and MHC expression levels, this study once again stresses the importance of individual MHC diversity in parasitic infections in sticklebacks. Even if the outcome of parasitic infection is mainly under control of the innate immune system, the strong link between innate and adaptive

immunity mediated by MHC genes (Dixon and Stet 2001) opens up perspectives for natural selection acting on MHC genes themselves as well as their expression levels.

Stocking of enclosures

Enclosures in the Große Plöner See

Photo: Martin Kalbe

CHAPTER IV

MHC optimality in real life

Abstract

Superior parasite resistance of the three-spined stickleback *Gasterosteus aculeatus* possessing an intermediate number of MHC class IIB alleles has been demonstrated by experimental studies and epidemiological field surveys. Combining the advantages of both approaches we used enclosure cages to expose 14 families of sticklebacks with known MHC genotypes to their natural parasite fauna and therefore test for MHC-optimality in a near field situation. After a mass die off caused by the extreme temperatures in August 2003, which killed 78% of the 277 fish used, we harvested the experiment. Survival differed significantly among families ($\chi^2_{(d.f.=1,\ n\ =\ 277)}$ = 42.7, P < 0.001) - a pattern that was mainly caused by length, a reasonable indicator of crowding during growth (R^2 = 0.813, $F_{1,12}$ = 52.175, P < 0.0001). Within the segregating sibships (range of alleles per individual: 3- 9) those fish with an intermediate number of MHC class IIB alleles surived best (R^2 = 0.364, $F_{2,30}$ = 8.601, P = 0.001). Fish with an intermediate number of MHC class IIB alleles also tended to have the lowest parasite load. Together with the negative effect of parasite load on growth ($F_{1,40}$ = 9.029, P = 0.005) this finding suggests a link between parasite load and mortality and thereby between allele number and fitness. Secondly, we were interested in whether families with segregating MHC class IIB genotypes have a selective advantage over those with monomorphic, non-segregating MHC genotypes (all genotypes with 4 alleles). Parasite loads, however, did not significantly differ between segregating and non-segregating families. The variation in parasite load was however significantly higher in non-segregating families ($F_{30,22}$ =

2.183, P = 0.031). We can thus confirm earlier findings and establish new links between MHC optimality and survival.

Introduction

Parasitism is the most abundant life style on earth (Windsor 1998) and its profound effect on host fitness renders parasites as one of the most prominent forces driving evolution. The need for hosts to counter parasite attack by developing resistance is the fuel of an arms race, that may be symbolized by the metaphor of the Red Queen (Van Valen 1973). One possible adaptation to parasitism is the maintenance of genetic diversity in defense genes to guarantee survival of some genotypes. One of the most striking examples of selectively maintained genetic polymorphism is the vertebrate major histocompatibility complex (MHC). In the vast majority of taxa studied so far the extensive polymorphism observed could be attributed to diversifying natural selection on the DNA sequence level (Apanius et al. 1997; Bernatchez and Landry 2003; Garrigan and Hedrick 2003). The cause and mode of selection however remain a matter of debate. The function of MHC molecules in presenting non-self peptides to T cells suggests that pathogens are the ultimate cause driving MHC evolution. In fact

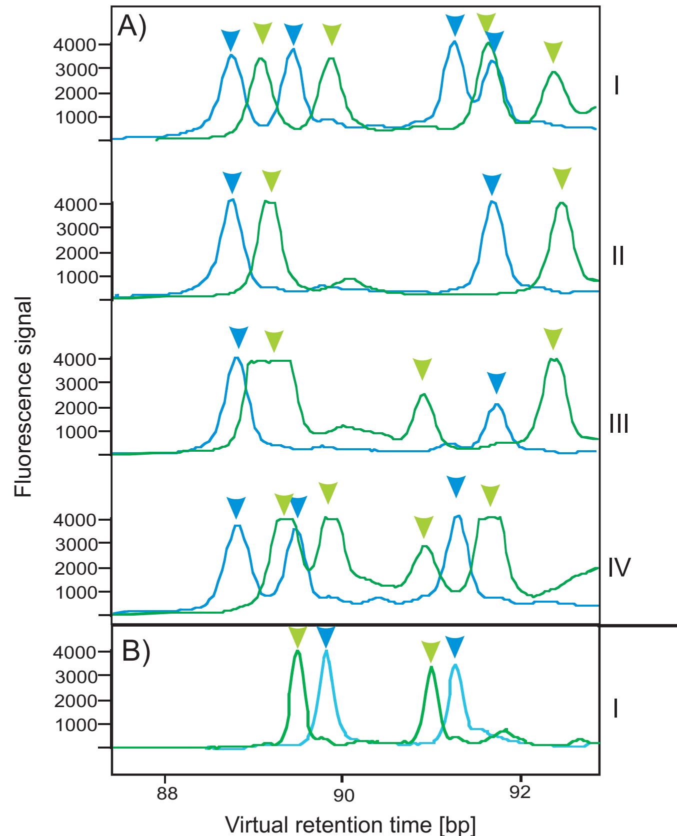


Figure IV-1: Examples of MHC class IIB SSCP genotypes. Blue peaks indicated PCR products generated with standard primer set, while green peaks show those from the modified primer set. A) shows the four genotypes found in segregating family 126x151. Individual MHC diversity ranges from four alleles in genotype III to eight alleles in genotype I . B) shows the only MHC class IIB genotype found in the non-segregating family 198x223 with four alleles.

several studies showed that certain MHC alleles are linked to either resistance or susceptibility against pathogens (Briles et al. 1977; Hill et al. 1991; Decamposlima et al. 1993; Godot et al. 2000; Langefors et al. 2001; Grimholt et al. 2003). Such associations are a prerequisite for balancing selection through pathogens either by overdominant selection/heterozygote advantage or frequency dependent selection (Apanius et al. 1997; Penn et al. 2002). Heterozygote advantage could explain balanced MHC polymorphism in the face of multiple parasite infections (Penn et al. 2002; McClelland et al. 2003; Wegner et al. 2003a, Chapter II; Wegner et al. 2003b, Chapter I; Kurtz et al. 2004; for review see Wegner et al. in press). Out of these studies the ones using three-spined sticklebacks *Gasterosteus aculeatus* as model system (Wegner et al. 2003a, Chapter II; Wegner et al. 2003b, Chapter I; Kurtz et al. 2004), (1) were based on animals from natural populations and (2) showed minimal parasitation at intermediate levels of individual MHC diversity (i.e. number of MHC class IIB alleles) – a pattern which also reflects female MHC based mate choice (Reusch et al. 2001a; Aeschlimann et al. 2003). The first evidence for an optimal intermediate MHC diversity stems from an epidemiological field study (Wegner et al. 2003b, Chapter I), in which age of fish, time of exposure to parasites as well as exposure dose could not be controlled. This correlational finding was later confirmed by an experimental study using three of the most abundant parasites in controlled infections protocols (Wegner et al. 2003a, Chapter II). Despite of their power to detect causalities, experiments can only mimic the natural situation to a certain extent and tend to amplify the controlled effect (Sorci et al. 1997). To test for MHC optimality in a more natural, yet controlled setting, we wanted to combine the advantages of a field study (natural parasite fauna, ambient abiotic factors) with the advantages of an experiment (i.e. controlled MHC genotypes, known exposure dose, randomization with respect to treatment) by using enclosure cages. The cages were stocked with 14 lab reared, parasite free fish families, which varied in their degree of MHC diversity (Fig. IV-1A). Some of them originated from MHC homozygous parents resulting in only one MHC genotype (non- segregating, see Fig. IV-1B) in all the offspring, while parents of other families were MHC heterozygous and their offspring had one of up to four different MHC genotypes (segregating). First of all, we were interested in potential MHC optimality within the MHC segregating families. Following earlier results (Wegner et al. 2003a, Chapter II; Wegner et al. 2003b, Chapter I; Kurtz et al. 2004) we would predict to find a minimal parasitation at an intermediate number of different MHC class IIB alleles (i.e. 5 –6). Additionally, we were interested

Table IV-1: Macroparasitic infection of surviving fish within the 14 families used. Families are characterized by their MHC genotypes (segregating / non-segregating) and the number of alleles of each genotype. Ranges of infection intensity per family are given for each parasite species.

Family (hatching date)	MHC genotype (number of alleles for each genotype)	N identified & dissected	NEMATODES Anguillicola crassus	Contracaecum spec.	Camalanus lacustris	Raphidascaris spec.	DIGENEAN TREMATODES Diplostomum pseudospathacaeum	Apatemon cobitidis	Tylodelphis clavata	Cyathocotyle prussica	Echinocasmus spec.	CESTODES Proteocephalus filicollis	Vallipora campylancristrota	CRUSTACEANS Ergasilus spec.
314x38 (06.06.)	Variable (5,8)	8	0-1	0-2	2-9	0-2	27-95	0-5	0-4	0-35	4-13	0-8	0-5	0-1
290x95 (07.06.)	Variable (4,5,6,8)	6	0-2	0-1	1-6	0	36-58	0-2	0-11	5-84	7-17	1-5	0	0-18
198x223 (28.04.)	Non-variable (4)	11	0-4	0-1	0-9	0-4	0-88	0-12	0-6	0-33	4-34	0-10	0-2	0-4
70x133 (05.05.)	Non-variable (4)	7	0-1	0-1	1-4	0-2	40-119	0-15	0-24	0-94	10-41	0-4	0-6	0-2
59x58 (21.05.)	Non-variable (4)	9	0-2	0-1	0-6	0-4	26-176	0-19	0-4	0-58	8-25	0-6	0	0-8
220x211 (15.05.)	Variable (4,8)	0	-	-	-	-	-	-	-	-	-	-	-	-
90x1 (03.05.)	Variable (6,7,8)	1	0	0	0	0	72	2	5	6	8	0	0	4
204x113 (02.05.)	Non-variable (4)	5	0-3	0	0-11	0-2	30-96	0-3	2-9	0-12	8-12	0-4	0-1	0-9
36x183 (25.04.)	Variable (3,4,5,6)	3	0-1	0-1	0-6	0-1	23-106	0-1	0-4	3-55	10-20	2-5	0-1	2-4
188x187 (02.05.)	Variable (4,6,7,8)	1	1	0	7	1	43	1	1	5	10	0	0	8
126x151 (04.0.5)	Variable (4,5,7,8)	2	1	1	1-8	0-1	58-62	0	2-4	2-90	14-16	0-1	0	0-4
50x43 (24.04.)	Variable (5,6,8,9)	1	0	0	6	0	111	1	1	13	15	3	2	0
218x205 (23.04.)	Variable (3,4,5,6)	3	0-2	1-3	0-1	0-2	45-75	1-2	0	5-27	5-15	2-9	0-1	0-1
55x66	Variable (4,6,7,8)	1	0	2	1	0	36	6	2	6	10	8	1	2

in differences between segregating and non-segregating sibships. It has been suggested that if selection favours MHC optimality, populations should consist of monomorphic (i.e. non-segregating) genotypes (Hedrick 2004). Regardless of how realistic these assumptions were (Wegner et al. 2004), it would be worth investigating whether there are selective advantages for individuals origninating from segregating families over those from non-segregating ones.

Another advantage of using outdoor enclosures is that conditions are likely to be less beneficial than in the lab, where usually food supply is sufficient and abiotic conditions are held constant. Therefore, mortality might be higher and direct effects on fitness might be more pronounced. Especially in a year like 2003, when northern Europe suffered from an extraordinary heat wave (Schär et al. 2004) consequences of unfavourable circumstances were probably amplified. Such consequences can also arise from, or at least interact with parasitemia and we would

therefore expect that costs of parasitation might eventually lead to death. Since parasitation is linked to MHC diversity, a direct link between indivdual MHC diversity and mortality can be expected.

Material and methods

Fish families

To stock enclosure cages we used fish from 14 lab-reared families. We chose families on the basis of their MHC genotypes. To determine genotypes before starting of the experiment we split each fertilized clutch of eggs in half and used 16 eggs of the first half for DNA extraction and genotyping, while we kept the other half in an aeriated glass jar until hatching. Fry was reared in one tank per family for the first weeks before distributing fish among several tanks to enhance growth. Each family contributed 20 individuals, exept for family 220x211 where only 17 individuals were available at the start of the experiment. Four families were sired by parents homozygous at the MHC loci, which resulted in offspring with an identical MHC genotype (Tab. IV-1,Fig. IV-1b) while members of the remaining 10 families differed in the degree of individual MHC diversity ranging from three to nine different MHC class IIB alleles (Tab. IV-1, Fig. IV-1a). Before stocking fish were weighed, measured and a spine was clipped for DNA extraction. Fish length ranged from 37.0 mm to 65.0 mm and weight ranged from 0.548 g to 2.483 g.

Genotyping

We genotyped each fish for its MHC class IIB alleles. To this end we applied single stranded conformation polymorphism (SSCP) on a 120 bp PCR product covering the antigen binding region of the exon 2 of the MHC class II β chain (Binz et al. 2001; Reusch et al. 2001a). We used a combination of two primer pairs that cover up to 80% of the variation in alleles present (Wegner et al. 2003b, Chapter I). Figure 1 shows examples of genotypes found in two families. To genetically tag and follow individuals over time, we also genotyped all fish before the start of the experiment and all survivors after its end on five polymorphic microsatellite loci (Largiader et al. 1999).

Enclosures & Dissection

We used five stainless steel net cages measuring 1 m * 0.5 m * 0.5 m with mesh size of 5 mm as enclosures. Fish families were evenly distributed among all cages (i.e. 4 fish/family and cage) resulting in 55-56 fish per cage. On April, 30th 2003 the cages were placed in a pentagram orientation at a depth of ≈1 m in the Große Plöner See, which is the lake where the parents of the fish originated from. Cages were checked and cleaned from algae fortnightly to enable immigration of infective parasite stages into the cage. Due to extreme temperature during August lake water levels dropped dramatically. To prevent oxygen depletion, we moved the cages deeper into the lake.

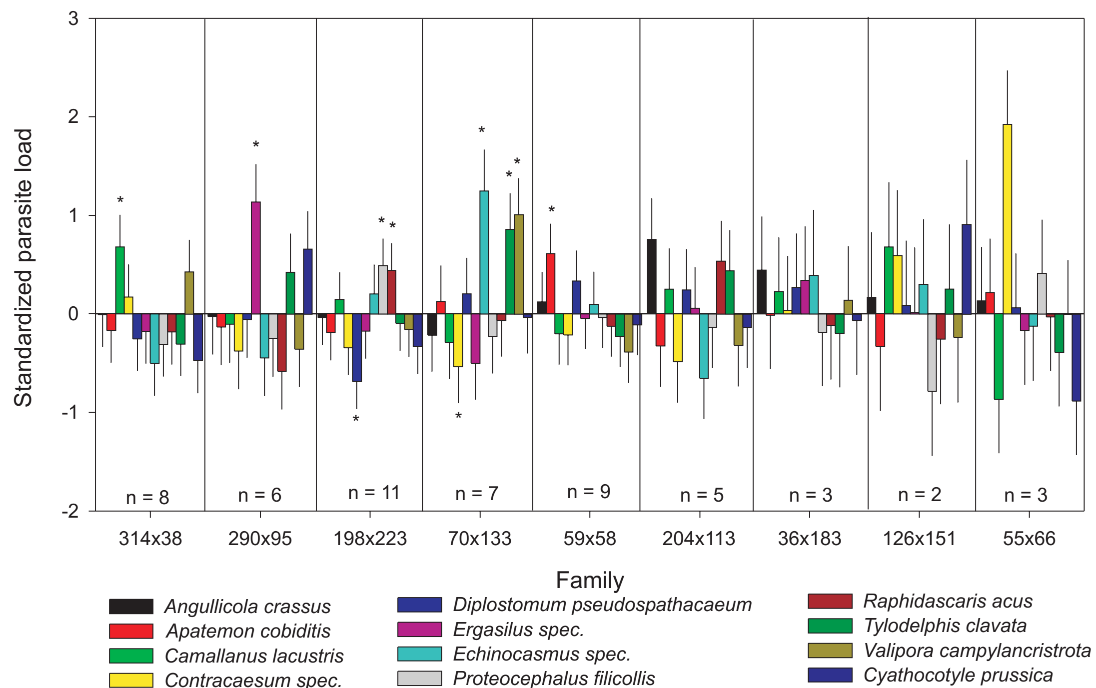


Figure IV-2: Standardized parasite load (LS mean from whole Repeated Measures model ± S.E.) from each parasite species within families with at least two survivors. * indicates significant interaction terms between parasite species and family in the whole Repeated Measures model.

This could however not prevent a mass die off, in which 78% of the fish died in between two checking intervals in late August. The remaining 61 fish were then brought to the lab, where they were dissected during the following weeks. Since the termination of the experiment came rather by surprise, dissection could not take place immediately. This does not impose problems for the counting of most of the parasites, which are likely to stay in the host waiting for transmission to the next host. We did, however, refrain from counting monogeneans like *Gyrodactylus gasterostei* because they

reproduce on the host and reproduction dynamics are surely influenced by the change in temperature regime. For counting the remaining parasite species we killed the fish in an excess solution of MS 222 and followed a slightly modified dissection protocol used by Kalbe et al. (2002).

Statistical analysis

Parasite loads

All tests were performed in JMP professional 5.0.1 (SAS Institute). Since each individual was measured several times for the occurrence of each macroparasite species, we used Repeated Measures MANOVA to test for significant differences in infection for each species. Counts of parasite species did however vary considerably between species. Therefore we used standardized count values for this analysis. The repeated measures model included cage and family plus all interactions with the repeatedly measured variable parasite species as factors (Tab. IV-2). Because the Mauchly criterion of the sphericity test was passed (Mauchly criterion = 0.1304, $\chi^2_{(d.f.=65)}$ = 65.468, P = 0.460), we used univariate tests with adjusted degrees of freedom to determine significant interaction terms of parasite species and family (Fig. IV-2).

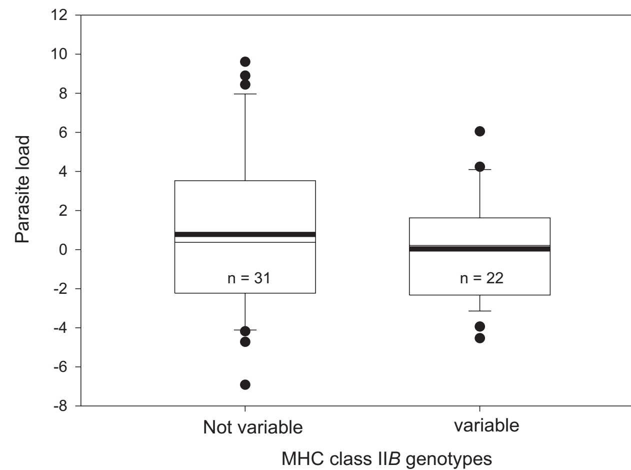


Figure IV-3: Box plots of overall parasite load expressed as the sum of standardized intensities from the 12 macro-parasite species found. While the mean does not significantly differ between the two groups, non-segregating MHC genotypes show significantly larger variation than segregating genotypes ($F_{30,22}$ = 2.183, P = 0.031).

For the analysis within families we used the sums of all standardized count values of all observed parasite species to get a measure of overall parasite load. This measure correlated well with the number of parasite species infecting the host (R = 0.656, n = 51, P < 0.0001, used by Wegner et al. (2003b, Chapter I)) but also takes the abundance of each single species into account. Due to the low sample size of each genotype we did

not look for allele/haplotype specific resistance against or susceptibility for each parasite species.

Survival

We analyzed the proportion of survivors using χ^2 tests. Significance in tests involving continuous response variates were analysed by means of ANOVA or ANCOVA, depending on the model used. For the within family test of the different MHC genotypes we used only those families with segregating MHC genotypes. We calculated residual survival probabilities of each MHC genotype by subtracting the average survival probability of the whole family from the survival probability of the respective MHC genotype within the family. If a MHC genotype survived better than others it would get a positive score, while a MHC genotype with lower survival would get a negative one. If intermediate individual MHC diversity is also advantageous in the context of survival, we would thus expect a higher proportion of intermediate genotypes with positive values.

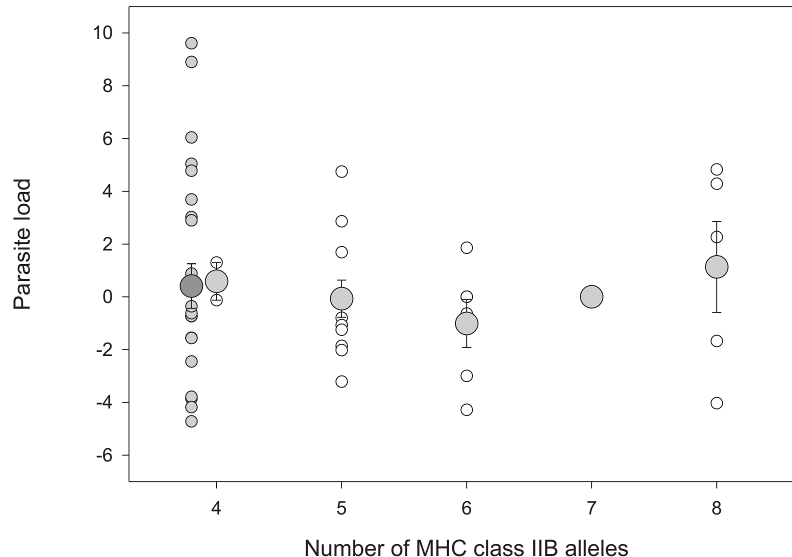


Figure IV-4: Mean overall parasite load (± S.E., large circles) and individual parasite loads (small circles) of surviving fish. Parasite load is expressed as the sum of standardized intensities from the 12 macro-parasite species found as a function of individual MHC diversity within segregating (light grey) and non-segregating (dark grey) families. Fish with six alleles show lower overall parasite load. Only 28 surviving fish entered the analysis and therefore the pattern is not significant. Nevertheless, the genotype with lowest parasite load matches the predicted optimal MHC diversity from previous studies (Wegner et al. 2003a, Chapter II; Wegner et al. 2003b, Chapter I; Kurtz et al. 2004). Since genotypes of segregating families with four alleles and non-segregating families are virtually identical in terms of parasite load, immunogenetic diversity seems to be predictive for parasite load.

Results

Out of the 61 fish recovered from the cages 58 could be unambiguously identified with the microsatellite markers. The three remaining fish were left out of the analysis.

Parasite load

Tab. IV-1 shows the range of intensities for each of the twelve macro-parasite species found in the surviving fish. We chose to present ranges instead of means, because sample sizes were too small within some families to calculate means. As we were also interested in family effects on infection we used a Repeated Measures MANOVA on the standardized parasite load of each species. Only families with two or more survivors entered the test. We found significant effects of the cage*parasite as well as the family*parasite interaction term (Tab. IV-2). Since significant differences among cages were found for some of the parasite species, we used least square means in Fig. IV-2 to show the detailed analysis of family*parasite interactions and thereby controlling for the cage effect. Infection by one parasite species did not seem to influence infection by any other species. In fact, out of the 66 pairwise comparisons only one (*Diplostomum pseudospathacaeum* vs. *Aparthemon* spec. R = 0.6253, P <0.0001) remains significant after Bonferroni correction for multiple testing. Therefore, infection with each species can be regarded as an independent effect.

Table IV-2: Repeated Measurement MANOVA for the sums of standardized infection intensity of each parasite species (i. e. total parasite load). Interaction terms with the repeatedly measured variable “parasite” show significant differences of species-specific infection intensities between cages and families.

Repeated measures MANOVA					
Between Subjects			*Exact F*	*DF*	*P*
	Population		0.4278	4,36	0.7553
	Family		0.3243	8,36	0.9513
Within subjects			*Approx. F*	*DF*	*P*
	All interactions	Roy’s max root	3.9129	12,36	< 0.0001
	Parasite	F test	0.2132	11,26	0.9949
	Parasite*cage	Roy’s max root	8.086	11,29	< 0.0001
	Parasite*family	Roy’s max root	4.4474	11,33	0.0004

As a measure of total parasite load we used the sums of all standardized parasite loads from each species. Overall there was a negative effect of total parasite load on the growth of the fish. Growth was calculated as ln(length dissection)- ln (length start). Fish

that had a higher total parasite load grew more slowly (Growth = 0.228 – 0.011 * total parasite load, $R^2 = 0.1842$, $F_{1,40} = 9.0289$, $P = 0.0046$) – an effect that was independent of initial length.

Total parasite loads did not significantly differ between families despite a comparatively wide range of means (2.871 ± 1.863 in family 70x133, -0.7279 ± 1.473 in family 314x38, $R^2 = 0.4634$, $P = 0.8743$). When comparing families with segregating and non-segregating MHC genotypes, the mean total load of non-segregating families is just marginally higher (Fig. IV-3). The variation within families with non-segregating MHC genotypes is however significantly higher than the variation within families with segregating MHC genotypes (Fig. IV-3, $F_{30,22} = 2.183$, $P = 0.031$).

MHC optimality has so far been demonstrated in terms of different parasite resistance between genotypes of segregating families (Wegner et al. 2003a, Chapter II). Parasite load can, however, only be estimated in surviving fish. Out of the 61 survivors only 28 came from segregating families, which is probably a too little sample size to detect a significant effect. Nevertheless, Fig. IV-4 shows that fish from segregating families with an optimal individual MHC diversity (i.e. 5-6 alleles) tend to have the lowest total parasite load, albeit not significantly ($R^2 = 0.0767$, $F_{2,18} = 0.9149$, $P = 0.4153$). Furthermore, fish with four alleles in a segregating family have a comparable parasite load to those of non-segregating families (Fig. IV-4).

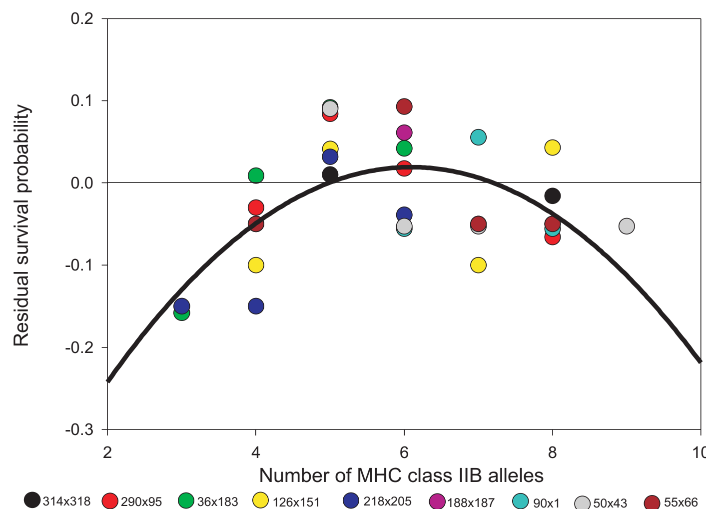


Figure IV-5: Relationship between number of MHC class IIB alleles and residual survival probability. Residual survival probability was calculated as the difference between survival probability of a certain genotype and the overall survival probability of the family. The quadratic polynomial (survival = -0.5595 + 0.1899 * $N_{alleles}$ – 0.0156 * $N_{alleles}^2$, $R^2 = 0.364$, $F_{2,30} = 8.601$, $P = 0.001$) shows that within segregating families fish with an individual MHC diversity of 6.086 MHC class IIB alleles have highest chance of survival.

Survival

Within segregating families

The residual survival probability of each MHC genotype in respect to the family survival follows a quadratic polynomial relationship (Fig. IV-5, survival = - 0.5595 + 0.1899 * $N_{alleles}$ – 0.0156 * $N_{alleles}^2$, R^2 = 0.364, $F_{2,30}$ = 8.601, P = 0.001). Maximal survival described by this function can be found at 6.086 alleles, which matches the previously observed MHC optima of 5.2 alleles (Wegner et al. 2003b, Chapter I), 5.8 alleles (Wegner et al. 2003a, Chapter II) and 6.2 alleles (Kurtz et al. 2004). Since the analysis included all segregating families with the exception of family 220x211, which had no survivors, this result might be biased by outliers from families with only single survivors. Excluding these from the analysis did neither dramatically change the significance of the result nor the point of maximal survival (R^2 = 0.487, $F_{2,15}$ = 7.1163, P = 0.0067, maximal survival at 6.18 alleles).

Segregating vs. non-segregating families

There were considerable differences in the proportion of survivors between the families used (Fig. IV-6, $\chi^2_{(d.f.=1, n = 277)}$ = 42.7, P < 0.001). Numbers of survivors ranged from 11 (= 55%) in family 198x223 to 0 (=0%) in family 220x211. At first sight it seems as if the non-segregating families (dark bars in Fig. IV-6) survive better than the MHC segregating families. This finding is, however, confounded by the length of the fish. Length did not significantly correlate with age of the fish (R = 0.257, n = 14, P = 0.3743). Fig. IV-7 shows that families with shorter fish survived significantly better than longer fish (R^2 = 0.813, $F_{1,12}$ = 52.175, P < 0.0001). It is probably a coincidence that all families with non-segregating MHC genotypes belonged to the shorter fraction.

Discussion

The fact that three-spined sticklebacks with an intermediate rather than a maximal number of MHC class IIB alleles are superior in terms of parasite resistance has been shown by two experimental studies (Wegner et al. 2003a, Chapter II; Kurtz et al. 2004) and one epidemiological field survey (Wegner et al. 2003b, Chapter I). We now tried to verify the results of these studies in the more natural, yet controlled setting of outdoor enclosures. The use of enclosures enabled us to evaluate the selective impact of the whole parasite fauna and its relation to individual MHC genotypes (Fig. IV-4, 7).

Furthermore, we were able to address new questions like MHC-dependent survival and could relate the optimal individual MHC diversity to maximal surivival within families (Fig. IV-5). By using within family comparisons we excluded other genetic factors, which were not linked to the MHC class II region, because the genetic background from other linkage groups should be randomized.

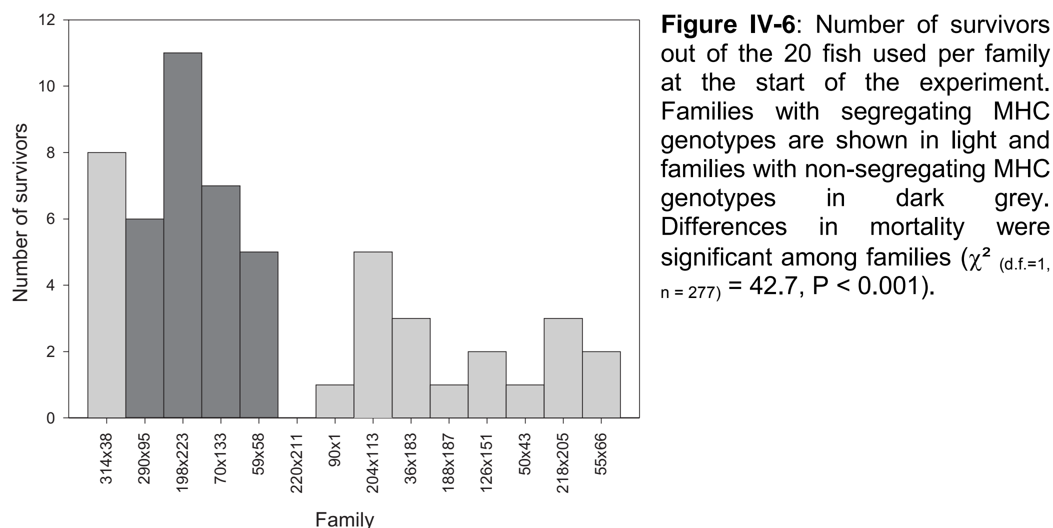


Figure IV-6: Number of survivors out of the 20 fish used per family at the start of the experiment. Families with segregating MHC genotypes are shown in light and families with non-segregating MHC genotypes in dark grey. Differences in mortality were significant among families ($\chi^2_{(d.f.=1, n=277)} = 42.7$, $P < 0.001$).

Parasite intensities found within survivors were unusually high when compared to other field data (Kalbe et al. 2002). Average eye fluke (*Diplostomum pseudospathacaeum*) intensity for example was 15-fold higher in the cages than in local populations (Tab. IV-1). It is uncertain if high intensities were caused by cage artifacts or can be attributed to the extreme climatic conditions (Schär et al. 2004). Due to the relatively enclosed space fish were kept in, cercariae released within a cage might encounter a fish with higher probablility than in the open water – probably resulting in higher infection intensities. A similar argument can be constructed for trophically transmitted parasites such as nematodes or cestodes. These are mainly transmitted by copepod first intermediate hosts and chances of being caught by a stickleback might be higher within a cage than outside. In this way the negative effect of parasitism on host health might be amplified.

On the other hand, the high intensities need not to be the result of extraordinarily high host-parasite encounter rates. Heavily parasitized individuals get preyed upon more easily (Crowden and Broom 1980; Owen et al. 1993). Exclusion of predation within cages might therefore enable survival of heavily parasitzed fish, which otherwise would have faced death in terms of predation.

Looking at each parasite species separately, we found significant differences in infection rates among different families (Fig. IV-2, Tab. IV-2). Since fish from one

family were distributed evenly among all cages such a result cannot be explained by cage effects. It rather suggests family effects or other genetic components. There was no superior family, which was most resistant against a majority of parasite species. Rather single families were resistant against one species, while being more susceptible against others (Fig. IV-2). This indicates the presence of genotype x genotype interactions, which might be expected from individual MHC genotypes associated to disease. To this end, we can say that the individual MHC diversity (i.e. the number of MHC class IIB alleles within an individual) is an important trait for the overall parasitic infections (i.e. sum of standardized single parasite species loads). Within the few surviving fish, those with six MHC class IIB alleles had the lowest parasite burden (Fig. IV-4). Even though this finding was not significant due to the small number of survivors, it supports earlier findings on MHC-dependent parasetimia in sticklebacks (Wegner et al. 2003a, Chapter II; Wegner et al. 2003b, Chapter I).

We were further interested in differences among families with segregating and non-segregating MHC genotypes. If there is a selective advantage of individuals with intermediate individual MHC diversity, it has been suggested that stabilizing selection should have deselected sub-optimal genotypes and populations should be monomorphic with respect to the number of individual MHC alleles (Hedrick 2004). Despite of some unrealistic assumptions implicit in these calculations, the relative performance of families originating from MHC-homozygous parents compared to those with segregating MHC genotypes is nevertheless interesting.

Mean total parasite load was slightly higher in families with non-segregating MHC genotypes but did not vary significantly between segregating and non-segregating families (Welch ANOVA for unequal variances, $F_{1,45.165} = 0.1307$, $P = 0.7194$). The variance in non-segregating families, on the other hand, was significantly higher (Fig. IV-3, F-test, $F = F_{30,22} = 2.183$, $P = 0.031$). Because families with segregating MHC genotypes are less likely to suffer from extreme infection rates, the environment in terms of parasite infections will be more predictable within such families. Mate choice in sticklebacks is correlated with the MHC genotypes of the two partners in such a way that females choose males to complete their own set of MHC class II alleles with those of the male to reach the optimal diversity of 5-6 alleles in their offspring after meiosis (Reusch et al. 2001a; Aeschlimann et al. 2003). This strategy provides the best possible MHC correlated parasite resistance in offspring (Wegner et al. 2003a, Chapter II; Wegner et al. 2003b, Chapter I; Kurtz et al. 2004). It would also disfavor MHC

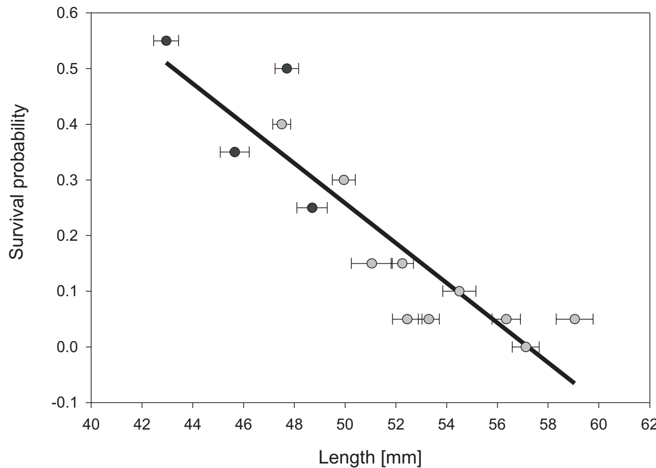


Figure IV-7: Proportion of survivors per family in relation to the mean length at the start of the experiment (± S.E.). Dots in dark grey indicate families with non-segregating MHC genotypes, while dots on light grey mark families with segregating MHC genotypes. It becomes obvious that the length of a fish, which can be approximated with age, is determining survival probability under extreme environmental stress (R^2 = 0.813, $F_{1,12}$ = 52.175, P < 0.0001) rather than MHC variability, which only coincidently correlates to length.

homozygotes because these genotypes do not segregate into different alleles, which would in turn result in over-proportional representation of sub-optimal MHC genotypes.

By definition parasitism induces fitness costs for the host. Sticklebacks with higher parasite loads grow slower under experimental conditions (Wegner et al. 2003a, Chapter II) and display higher levels of unspecific cellular immunity (i.e. oxidative burst, Kurtz et al. 2004). Also, fish in this data set with a higher total parasite load grew slower (Growth = 0.228 – 0.011 * total parasite load, R^2 = 0.1842, $F_{1,40}$ = 9.0289, P = 0.0046). Even though females with lower body condition are less fecund (Wootton 1976), any measure of growth will only be a component of total fitness, which possibly is traded off against other traits like timing of reproduction (Stearns 1992). A more direct link that really demonstrates the advantages of MHC optimality for life-time reproductive success in sticklebacks is missing so far. Here, we can show that within families with segregating MHC genotypes, those genotypes with 5-6 alleles have the highest probability of survival (Fig. IV-5). Since natural selection is the struggle for life (Darwin 1859), survival before reproduction is an ultimate measure of fitness.

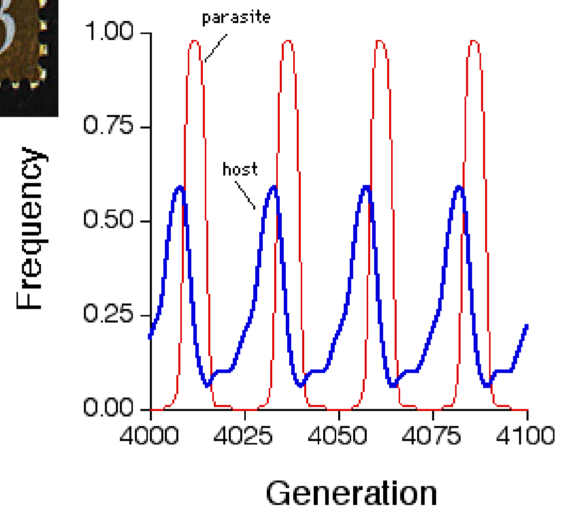
However, a lot of other factors will influence an individual's chance to survive. For the stickleback families used in our experiment their length at the start of the experiment was one of the main factors determining survival by explaining 81% of the variation found on family level (Fig. IV-7). Since length was not correlated to age, we think that length, which can be determined by the condition in early larval stages (McCormick and Hoey 2004) reflects stress caused by crowding in tanks during development. Smaller fish might have come from larger families and experienced more stress during early

larval growth, which might have caused higher stress tolerance and hence higher survival.

The fact that length is the main predictor of survival among families does not exclude the survival advantage of MHC genotypes of intermediate allelic diversity (Fig. IV-5), because this analysis was done within families. Within families variation in length between surviving and dead fish was comparatively small. Within the eight families with more than two survivors none had statistical significant differences between surviving and dead fish and only within three of these the difference was poiting into the predicted direction (sign test, $P = 0.375$).

We cannot directly relate parasitation to mortality in this study because infection data only exists for surviving fish. However, MHC-dependent parasite load in segregating families (Fig. IV-4) together with the negative effects on growth matches the pattern of survival within these families (Fig. IV-5). Individual MHC genotypes have been linked to survival in several studies, which identified protective alleles against fatal forms of disease (Hill et al. 1991; Godot et al. 2000; Langefors et al. 2001; Lohm et al. 2002; Grimholt et al. 2003). These studies focused on the protective quality of single alleles. Combining the outcome of these studies with the matching results for survival and parasite load presented here, it seems not to be far-fetched to impose a selective role of parasites in determining survival. Even though the link between parasite load and survival is not direct, the pattern of parasite infections and survival within families with segregating MHC genotypes suggests that there might be such a connection. Other stress factors like heat or oxygen depletion might be necessary in addition to the impact of parasites to reveal this pattern. Environmental stress has already been linked to strong selection on MHC genotypes (Cohen 2002). Since extreme heat waves like Europe witnessed in 2003 are getting more likely in the context of global warming (Schär et al. 2004), the reciprocal interaction of environmental, abiotic stress and parasitism, which is fuelled by the co-evolutionary arms race between parasites and hosts, will become an increasingly interesting field for addressing question of rapid adaptation and evolution.

Allice running with the Red Queen



Theoretical oscillations of host- and parasite genotypes

Source: C. Lively's homepage @ http://sunflower.bio.indiana.edu/~clively

CHAPTER V

Red-Queen type fluctuations of stickleback MHC class IIB alleles and macro-parasitic infections

Abstract

Parasite-driven balancing selection can maintain genetic polymorphism in defense genes by two mechanisms: overdominant and frequency dependent selection. A case in point are genes of the vertebrate's major histocompatibility complex (MHC). Overdominant selection on classical MHC genes was detected in multiple pathogen challenges in mice (*Mus msuculus*) and in three-spined sticklebacks (*Gasterosteus aculeatus*). The Red-Queen metaphor posits that the co-evolutionary arms race between hosts and parasites will lead to negative frequency dependence of fitness (i. e. resistance) conferred by single alleles/haplotypes. To test whether changes in MHC allele frequency correlate with parasite prevalence in sticklebacks, we analysed a time series of three consecutive generations of fish from three populations. We found significant frequency changes in seven out of a total of 41 MHC class IIB alleles. To evaluate the relative contribution of genetic drift, we examined frequency changes in seven polymorphic microsatellite markers and found significantly fewer alleles displaying temporal changes in frequency (3/45 alleles, χ^2 = 10.262, P = 0.0014). Seven of the eleven sampled macro-parasite species showed significant variation in prevalence within the three generations. In five parasite x population combinations, prevalence was associated with a particular MHC class IIB allele. Four MHC class IIB alleles conferred protection against infection while one was associated with increased susceptibility. This study provides correlative evidence for genotype-

genotype interactions - a prerequisite for negative frequency dependent selection - and reveals the connection between frequency changes within host MHC class IIB genotypes and parasitic infection as predicted by the Red-Queen theory.

Introduction

The “Red Queen hypothesis” was originally developed to explain how extinction of co-evolving species pairs may be avoided over evolutionary time scales, for example interacting predators and their prey or hosts and parasites (Van Valen 1973). Especially in host-parasite interactions, co-evolutionary arms races could lead to recurrent oscillations in genotype frequencies. Such cycles show the importance of genotype diversity, which in turn is only available because of recombination in sexual reproduction that leads to the formation of a nearly unlimited number of novel genotypes (Hamilton et al. 1990). These theoretical predictions imply specific genotype-genotype interactions between host and parasite genes, also known as matching alleles model (Howard and Lively 1994). According to this model, hosts have polymorphic recognition genes, whose alleles specifically recognize variable antigens derived from proteins that in turn are encoded by specific alleles of parasite genes. Allele frequencies at these loci should fluctuate antagonistically in a negative frequency dependent manner, with rare alleles conferring the highest fitness.

Due to their polymorphism and function in self/non-self discrimination, genes of the vertebrate’s major histocompatibility complex (MHC) are ideal candidates to investigate such frequency oscillations. The striking polymorphism of MHC genes is partly maintained by overdominant selection or heterozygote advantage in multiple pathogen challenges in mice (*Mus msuculus* (Penn et al. 2002; McClelland et al. 2003)) and three-spined sticklebacks (*Gasterosteus aculeatus* (Wegner et al. 2003a, Chapter II; Wegner et al. 2003b, Chapter I)). However, if hosts and parasites engage in co-evolutionary arms races as predicted by Red Queen theory, the fitness conferred by single alleles/haplotypes should vary over time in a negative frequency dependent fashion. The best documented example for negative frequency dependent selection in MHC genes comes from humans infected with Epstein-Barr virus (EBV). The MHC

class I allele HLA-*A11* confers resistance to infection with EBV only in populations where the allele is rare. In populations with high frequency of this allele, virus strains have fixed a mutation that prevents presentation of immuno-dominant epitopes by HLA-*A11* molecules (Decamposlima et al. 1993).

While this convincingly demonstrates the potential of frequency dependent selection, yet it remains a single point in time and cannot capture the predicted oscillations of MHC and virus genotypes. So far, only a single study attempted to look at frequency changes of MHC genes in natural populations. Westerdahl and coworkers found significant frequency variation between years for one MHC class I allele (B4b) in great reed warblers (Westerdahl et al. 2004a). Fluctuations in the allele frequency of B4b were not caused by genetic drift. The allele was associated to infection with avian malaria (Westerdahl 2003), and the authors argue that only those individuals possessing the allele were able to survive.

In this study, we used three-spined sticklebacks as a model system to investigate whether MHC class IIB allele frequencies change significantly between generations and whether or not these changes correlate to the prevalence of the most common macro-parasites. To test this hypothesis we caught fish from three different populations in three consecutive generations, sampled their parasite burden and identified their MHC class IIB genotypes. Even in genes under selection, such as the MHC, changes in allele frequencies are always subjected to genetic drift. We needed to quantify the putative effect of random factors on MHC allele frequencies using an independent marker set. To this end we genotyped all individuals at seven polymorphic microsatellite markers as well. If frequency changes are mainly caused by drift, then frequency differences between years should be similar for microsatellite and MHC class IIB alleles.

However, based on the importance of MHC class IIB genotypes for the outcome of parasitic infection as well as for mate choice decisions (Aeschlimann et al. 2003; Wegner et al. 2003a, Chapter II; Wegner et al. 2003b, Chapter I; Kurtz et al. 2004), we would expect the magnitude of parasite induced selection to outweigh the effects of genetic drift.

Material and methods

Sampling and dissecting fish

We sampled young of the year (0+) fish from three populations in northern Germany over a period of three years. We used the Schwale river (SCW) and the lake Vierer See (VS, described by Kalbe et al. (2002)) as sampling sites. These localities were chosen, because parasite faunas differ markedly between lake and river populations (Kalbe et al. 2002). We additionally chose a tributary flowing into VS named Vierer Graben (VG), because the parasite fauna here is somehow intermediate between typical lakes and rivers (M. Kalbe and K.M. Wegner, unpublished data) offering the opportunity to investigate the diversification of parapatric populations along a parasitation cline. Since 0+ fish reach maturity in the following year the three cohorts can be regarded as three consecutive generations. We sampled 25 fish per population and year, except for VG and VS in 2003, where 26 and 27 fish were sampled respectively, because within the first 25 fish caught some 1+ fish were found. These fish were excluded from the analysis, because we strictly wanted fish from the same generation within one sample. Furthermore, due to their higher age the likelihood of picking up more parasites and thereby biasing our results is increased.

Table V-1: MHC class IIB and microsatellite alleles with significant frequency changes between the three sampling generations. Since we performed one test for each allele we applied Bonferroni-correction. Critical P-values were $P_{crit\ (k=38)} = 0.0013$ for MHC class IIB alleles and $P_{crit\ (k=45)} = 0.0011$ for microsatellite alleles.

Population	***MHC***			***Microsatellites***		
	allele	χ^2	P	allele	χ^2	P
SCW	R01-B	18.361	<0.0001	GAC 1097-109	15.298	0.0005
	R04-B	18.922	<0.0001			
	R07-B	16.88	0.0002			
	R07-C	16.88	0.0002			
VG	R02-C	14.856	0.0006			
	R05-F	20.924	<0.0001			
	R08-A	14.552	0.0007			
VS	R05-F	21.5	<0.0001	GAC 1097-121	16.697	0.0002
				GAC 4170-100	17.266	0.0002

Fish were caught on November 1st 2001, December 12th 2002 and December 9th 2003 in SCW, November 5th.2001, November 26th.2002 and November 25th.2003 in VG and

VS. Dissection took place within two days after catching and followed a slightly modified protocol of Kalbe et al. (2002), which covers all main macroparasite species within these populations. During dissection a spine was clipped for later DNA extraction with the DNeasy tissue kit (Qiagen, Hilden).

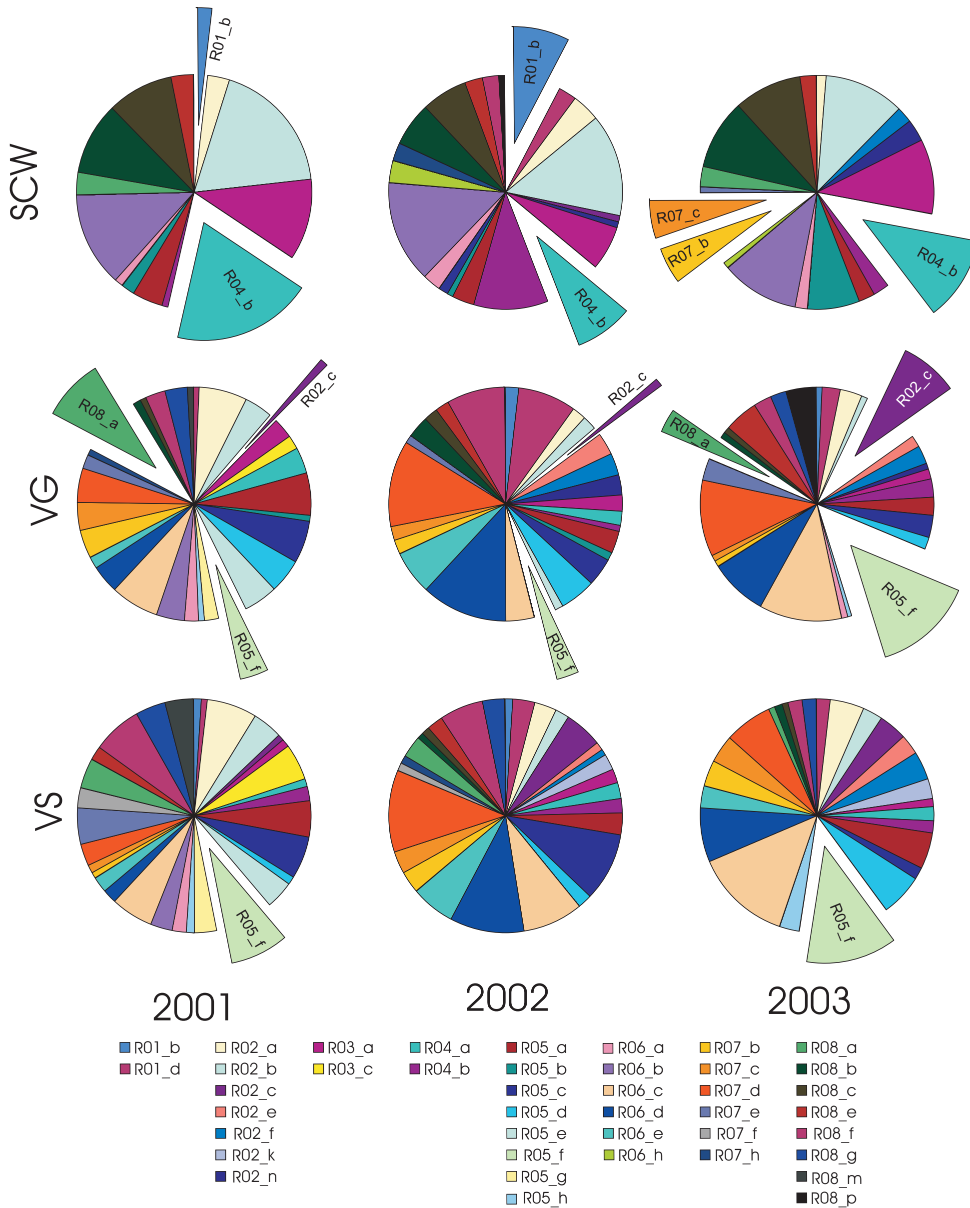


Figure V-1: MHC class IIB allele frequencies from three populations of three-spined sickleback over three consecutive years. Only alleles showing a frequency of ≥10% in any year and population were included. Those alleles with significant frequency changes over time (see Tab. V-1) within one population are highlighted.

Genotyping

The exact genomic architecture (e. g. number of loci) of stickleback MHC class II genes is unknown. Recent genomic data suggest that the age of MHC class II duplications is relatively young (Reusch et al. 2004), and that different loci exchange sequence motifs by inter-locus gene conversion (Reusch et al. 2004; Reusch and Langefors, in review). The resulting similarity between duplicated class II loci makes the design of single locus primers extremely difficult if not impossible (Reusch et al. 2004). Hence, we genotyped sticklebacks for their MHC class IIB genes using a motif-specific PCR strategy that does not discriminate among loci. This strategy also proved to be successful in passerine birds, where similar difficulties were encountered (Westerdahl et al. 2004b). This method is a considerable improvement for allelic discrimination compared to MHC class IIB genotyping techniques used before (Binz et al. 2001; Reusch et al. 2001a; Wegner et al. 2003b, Chapter I) because we felt that primer pairs used in these studies would not have sufficient resolution to discriminate all alleles. Therefore, we used the new primer sets amplifying a larger part of the exon 2 of stickleback MHC class IIB genes (T.B.H. Reusch, unpublished data). All primer pairs consisted of the same forward primer *GA11* 5'- AAC TCC ACT GAG CTG AAG GAC ATC -3', which was also used as a sequencing primer to generate the set of sequences out of which the motif specific reverse primers were designed. Sequences of reverse primers differed by at least two base pairs, preferably at the 3-prime end. Detailed sequences were *R01* 5'-CTT AGT CAG AAT ATT GTT GTA ATC -3', *R02* 5'-CGG ACT TAG TCA GCA CAT TG-3', *R03* 5'-AGT CAG CAT ATT GTT GTA CTC G -3', *R04* 5'-CAG AAT ATT GTT GTA ATA GAC CG -3', *R05* 5'-GTC AGC ATA TTG TTG TAC CAG-3', *R06* 5'-CGG ACT TAG TCA GAG CAT TG-3', *R07* 5'-CGG ACT TAG TCA GAG CAG CT-3', *R08* 5'-ATA TTG TTG TAA TCG ATC TGG A -3'. We used duplex PCR reactions combining R01 with R05, R02 with R06, R04 with R07 and R03 with R08, which were labeled with different fluorescent markers (6-FAM, HEX). Products were amplified in individual 20µl reactions using 2 µl of GA11 (5 µM) forward primer, 1 µl of each reverse primer (5 µM), 1 µl of $MgCl_2$ and 0.2 units of Taq polymerase (Promega). Cycling proceeded with an initial denaturation step at 94°C for 3" followed by 29 cycles of 0'30" denaturation at 94°C, annealing for 0'30" at 58°C and extension for 0'45" at 72°C. PCR products were separated by single stranded confirmation polymorphism (SSCP) using an ABI 3100 capillary sequencer (Applied

Biosystems, Darmstadt). Since all primer pairs amplified similar products, we could not exclude cross-amplification or mispriming. Cross-amplification of primers was minimized because reverse primers were selected to amplify sequences groups that are at least 3 bp different from one another (except for primer pairs R-03 and R-08 with only one bp difference at the 3'-end). Even in the latter case, mispriming in PCR reactions with single sequences from cloned PCR products as template resulted in 10-fold decrease in product amount (T.B.H. Reusch, unpublished data). Therefore, we scored genotypes conservatively only counting amplifications exceeding 10% of the maximum product amount from all four PCR reactions of each individual DNA preparation. The majority of sequences detected this way seemed to be expressed. Out of 42 SSCP signals detected in 6 individuals based on genomic DNA, 37 were also found in cDNA generated from spleen tissue.

Additionally, we genotyped all fish on seven polymorphic microsatellite loci (Largiader et al. 1999). For details on PCR recipes and amplification conditions see Reusch et al. (Reusch et al. 2001b).

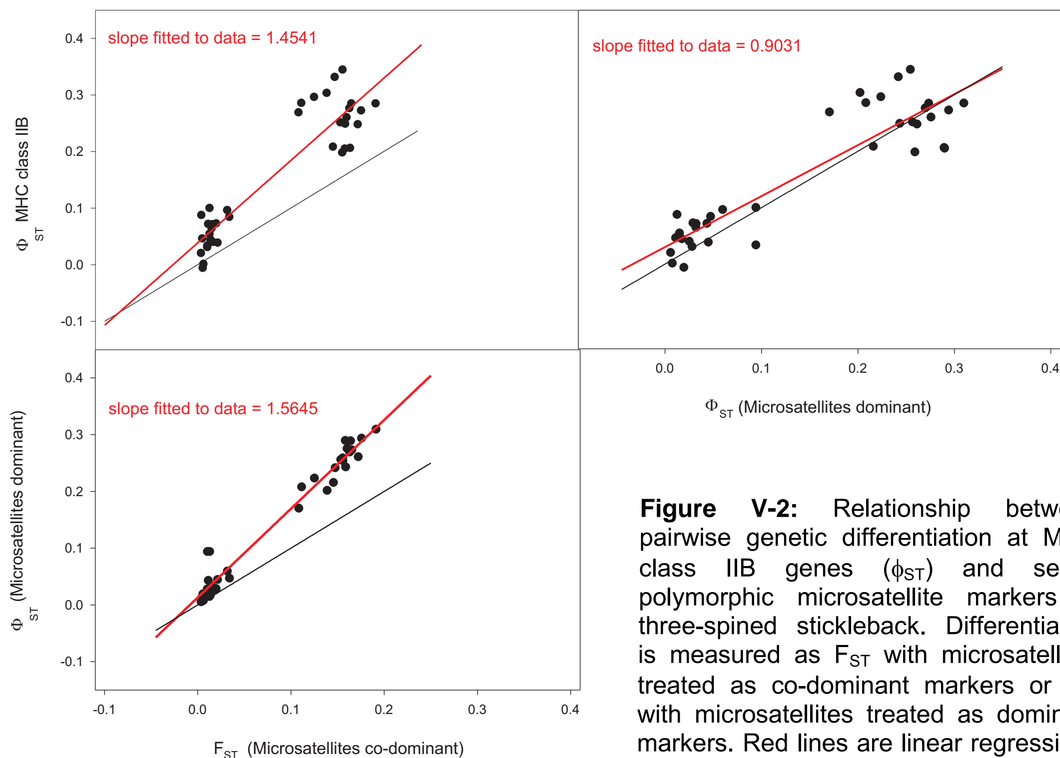


Figure V-2: Relationship between pairwise genetic differentiation at MHC class IIB genes (ϕ_{ST}) and seven polymorphic microsatellite markers in three-spined stickleback. Differentiation is measured as F_{ST} with microsatellites treated as co-dominant markers or ϕ_{ST} with microsatellites treated as dominant markers. Red lines are linear regressions fitted to the data while black lines indicate a slope of one. Panel A) shows the relationship between MHC class IIB and co-dominantly treated microsatellites. Panel B) shows the relationship between MHC class IIB and dominantly treated microsatellites. And C) shows the relationship between the two different scoring methods at the microsatellite loci.

Statistical analysis

First of all we were interested in temporal frequency changes of MHC class IIB alleles relative to frequency changes of selectively neutral microsatellite markers. We calculated pairwise genetic differentiation based on allele frequencies (F_{ST} for microsatellites and ϕ_{ST} for MHC alleles, respectively). We only included alleles, which showed a frequency of ≥ 10% in any one generation and population, because constantly rare alleles are obviously not of selective importance during the three generations sampled. Because we could not assign MHC alleles to particular loci we scored MHC alleles in a presence/absence fashion thereby treating each allele as a single locus. This is a substantial difference to co-dominant markers such as microsatellites, where every allele can be assigned to a single locus and homozygous loci get double allele counts (co-dominant scoring). Such a difference in scoring will substantially bias results because under dominant-recessive scoring, heterozygosities are substantially lower given only two alleles at any pseudo-locus (i.e. present absent). This, in turn, will lower the maximal differentiation under complete separation (Hedrick 1999). To examine this we also scored the microsatellite data analogous to the MHC alleles, i.e. constructing a presence/absence matrix for microsatellite alleles (dominant scoring).

Despite discarding the information contained in homozygotes, dominant scoring resulted in the highest correspondence of both measures. By analysing MHC class IIB and microsatellite alleles the same way, we increased the differentiation found at microsatellite loci and decreased the difference between the two markers. However, according to our hypothesis differentiation should be higher if measured with MHC alleles and any significant difference found between the markers despite reducing it will be conservative with respect to our hypothesis. Therefore, calculations were based on dominant scoring of microsatellites. We tested global genetic differentiation between generations to see whether differentiation within MHC genes exceeds that of the microsatellite loci. F_{ST} and ϕ_{ST} values were calculated using the software Genetix 4.0.1 (Belkhir et al. 1998) after formation of pseudo-diploid data for dominant scoring and significance was tested by comparing observed data to 1000 randomly permuted data sets. Confidence limits of ϕ_{ST} values were calculated by jack-knifing over 'loci'.

After determining significant genetic differentiation between years, we were interested in the alleles contributing most to overall differentiation, i. e. changes of MHC class IIB

allele frequencies that significantly deviated from random fluctuations. To this end we tested each allele within each population by logistic regressions using log-likelihood ratios as test statistic compared to a χ^2 distribution. Because we performed multiple tests we applied Bonferroni correction (Sokal and Rohlf 1995), which resulted in a critical P-value of $P_{crit\ (k=41)} = 0.0012$ for the 41 MHC alleles. A similar procedure was used for microsatellite alleles. Since more alleles were included in this test the Bonferroni-corrected critical P-value was set at $P_{crit\ (k = 45)} = 0.0011$.

These alleles were then subjected to association tests with those parasite species showing significant fluctuations in prevalence over the three years covered, again applying Bonferroni-correction for multiple testing. Significant associations between presence of alleles and infection were then determined by Fisher's exact tests. We applied two-tailed tests, because alleles can either confer resistance or susceptibility to parasitic infections. The direction of the association was determined by Spearman's rank correlation with a negative ρ symbolising protection while a positive ρ would signal susceptibility.

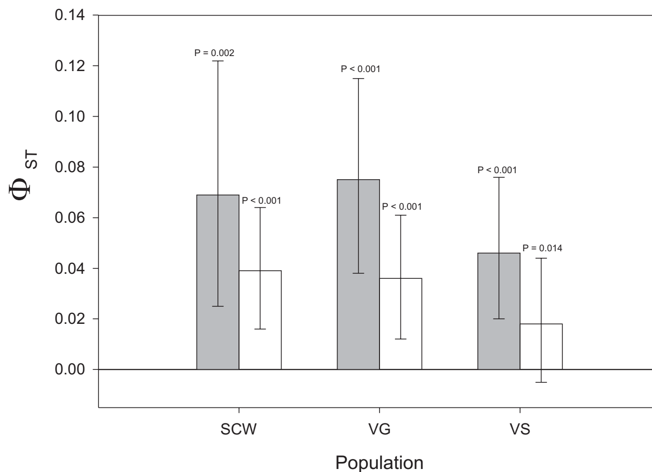


Figure V-3: Global ϕ_{ST} values for MHC class IIB (dark grey) genes and microsatellites (white, treated as dominant markers) within each population indicating significant genetic variation between years. Error bars display the 95% confidence interval around the mean. P-values were obtained from 1000 permutated data sets using the software Genetix (Belkhir et al. 1998).

Results

Parasites

Our parasite survey covered a total of 13 macro-parasite species in the 228 fish dissected. These were the nematodes *Anguillicola crassus*, *Contracaecum spec.*, *Camallanus lacustris* and a *Raphidascaris acus*. Furthermore, we found following digenean trematode species: *Diplostomum pseudospathacaeum*, *Cyathocotyle prussica*

and *Echinocasmus spec*. along with the monogenean *Gyrodactylus gasterostei*. Cestodes were represented by *Valipora campylancristrota*, *Paradilepis scolecina* and *Proteocephalus filicollis*. Of minor importance were mollusc parasites of the genus *Glochidia* and the protozoans *Trichodina spec.* and *Apiosoma spec.*. We found significant differences in prevalence between host generations in five of these species (Tab. V-2). These species comprise promising candidates to look for MHC class IIB alleles with similar fluctuations.

Table V-2: Significant frequency changes in parasite prevalence within the three populations and association to MHC class IIB alleles with significant frequency variation over time (Tab. V-1). Direction of association (i.e. protection or susceptibility) was determined by Spearman's rank correlation with a negative ρ indicating protection and a positive ρ indicating susceptibility.

Pop.	*Prevalence*						*Associated to*		
	Species	2001	2002	2003	χ^2	P	allele	ρ	P [§]
SCW	*Gyrodactylus gasterostei*	0.96	0.68	1	14.394	0.0007	R04-B	-0.2901*	0.0242
	Raphidascaris acus	0.24	0.28	1	46.757	<0.0001	R07-B	0.3166**	0.0115
VG	*Diplostomum pseudospathacaeum*	0.68	0.76	0.13	25.387	<0.0001	R05-F	-0.2721*	0.0237
	Cyathocotyle prussica	0.32	0.16	0	9.230	0.0099	-	-	-
	Gill metacercariae	0.6	0.48	0	21.882	<0.0001	R02-C	-0.2996*	0.0109
	Valipora campylancristrota	0.56	0	0.08	26.832	<0.0001	R05-F	-0.2698*	0.0284
VS	*Anguillicola crassus*	0.08	0.48	0.25	10.814	0.0045	-	-	-
	Gill metacercariae	0.44	0.44	0	15.028	0.0005	-	-	-
	Valipora campylancristrota	0.52	0.04	0.17	16.777	0.0002	-	-	-

[§] Fisher's exact test for overall association, * P < 0.05, ** P < 0.01

Genotypes

A total of 78 MHC class IIB alleles could be discriminated by our typing method. Out of these 41 alleles had a frequency exceeding 10% in any one population and generation. Fig. V-1 shows the relative frequencies of these alleles for all populations and generations. When looking at pairwise genetic differentiation it becomes obvious that the scoring method for the microsatellite data (dominant or co-dominant scoring) is essential (Fig. V-2). When comparing F_{ST} (co-dominant scoring) and ϕ_{ST} (dominant scoring) for microsatellite alleles, dominant scoring biases the amount of differentiation upwards (slope = 1.5645, Fig. V-2C). A similar pattern is found when ϕ_{ST}-values from MHC alleles were compared to F_{ST}-values from microsatellites (slope = 1.4541, Fig. V-

2A). The upward bias disappeared when both ϕ_{ST}-values from MHC and microsatellite alleles were compared (slope = 0.9031, Fig. V-2B) Here, the within-population between-generation comparisons of MHC genes and microsatellites were relatively higher (Fig. V-2B left set of values above the slope of one) than the between-population comparisons (Fig. V-2B, right set of values roughly equal to the slope of one). Interestingly, when summing up the differences of both slopes to a reference slope of one for the MHC data, the added difference was virtually identical to the difference of the slope between the two scoring methods of the microsatellites to the reference slope of one (0.551 vs. 0.565).

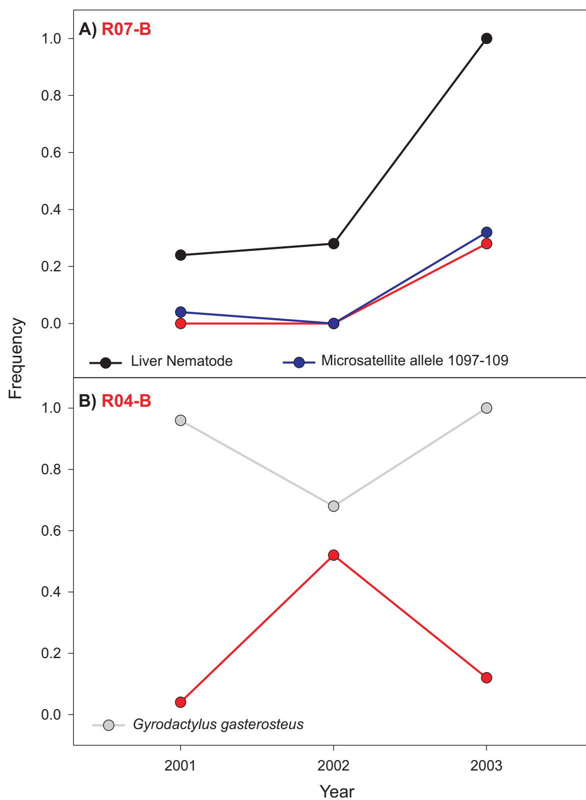


Figure V-4: Time course of MHC class IIB and microsatellite allele frequencies in three-spined sticklebacks and parasite prevalences, showing significant associations between infection and presence of the allele within individuals in the river Schwale (SCW). A) The black line shows the prevalence of the liver nematode, which significantly associated to the MHC class IIB allele R07-B (red line) and the microsatellite allele 1097-109 (blue line). B) The light grey line shows the prevalence of *Gyrodayctylus gasterostei* , which is significantly associated with the MHC class IIB allele R04-B (red line).

A global test for genetic differentiation within populations and between generations revealed significant differentiation at MHC genes as well as microsatellites (Fig. V-3), which indicates that genetic drift might indeed play a role within these populations. The small effective population sizes (N_e) in the populations accentuate the role of genetic drift. N_e-values calculated with NeEstimator (Peel et al. 2004) were 39.9 (95% C.I. 22.3 – 121.2) in SCW , 27.8 (95% C.I. 17.7 – 54.9) in VG and 64.6 (95% C.I. 33.9 – 315.7) in VS. The difference within pairs was, however, about 50% higher for the MHC class IIB alleles than for the microsatellites (Fig. V-3, paired t-test, mean difference 0.0246, t = 5.17976, d.f. = 5, $P_{2\text{-tailed}} = 0.0035$). Since the amount of genetic differentiation at MHC genes

exceeded the differentiation caused by genetic drift at the microsatellite loci, we proceeded to identify those alleles mainly causing differentiation in the global test. We identified seven MHC class IIB alleles with frequency changes deviating significantly from a random pattern (Fig. V-1, Tab. V-1) even after Bonferroni-correction. We also found significant frequency changes in three microsatellite alleles stemming from two loci (Tab. V-1). While this could indicate an important influence of genetic drift, the proportion of microsatellite alleles with significant variation was much lower than that of MHC class IIB alleles (MHC class IIB: 8 out of 41, microsatellites: 3 out of 45, χ^2 = 10.262, P = 0.0014)

Four out of the seven MHC class IIB alleles were significantly associated to prevalence of at least one of the fluctuating parasite species in SCW and VG (Tab. V-2). Four associations conferred resistance, while only one allele-parasite pair was associated with susceptibility (Tab. V-2). We did not find any significant association in VS. The frequency changes of MHC class IIB alleles and their associated parasite species can be seen in Fig. V-4 for SCW and Fig. V-5 for VG, respectively.

Discussion

We have previously shown that in natural populations of three-spined sticklebacks individual MHC class IIB diversity reduces parasitic infection through overdominant selection (Wegner et al. 2003b, Chapter I). MHC heterozygous individuals suffer less from multiple infections in sticklebacks (Wegner et al. 2003a, Chapter II; Kurtz et al. 2004) and mice (Penn et al. 2002; McClelland et al. 2003). Here, we identify another postulated selection process by providing correlative evidence for coupled frequency changes in stickleback MHC class IIB alleles and parasite infections associated with these alleles (Tab. V-2). Such fluctuations caused by negative frequency dependent selection are predicted by the Red-Queen theory (Van Valen 1973) but rely on the assumptions of matching allele models (Agrawal and Lively 2002).

While the mechanistic basis of negative frequency dependent selection on MHC genes was already shown for humans infected with the Epstein-Barr virus (Decamposlima et al. 1993), only one study attempted to look for MHC allele frequency shifts in natural populations (Westerdahl et al. 2004a). In Great Reed warblers *Acrocephalus arundinaceus*, one allele varied significantly in frequency between nine cohorts corresponding to only three generations. As in the present study, frequency changes in

MHC genes varied more strongly than the frequencies of microsatellite alleles, indicating that selection on MHC genes exceeded neutral genetic processes.

In our data set, the relative impact of genetic drift in the stickleback populations used here seems to be higher than in reed warblers. In addition to significant changes in MHC class IIB allele frequencies, three of the 45 microsatellite alleles also showed significant variation (Tab. V-1). This is consistent with the relatively small effective population sizes found in the sickleback populations of 28-65 individuals. The proportion of alleles with significant variation in microsatellites was however significantly lower than in MHC alleles (χ^2 = 10.262, P = 0.0014) – a pattern that is also supported by global genetic differentiation (Fig. V-2,3). Since MHC class II B genes are situated on a single linkage group, the excess of variation compared to the microsatellites stemming from seven unlinked loci cannot be explained by drift alone. Furthermore, we cannot exclude that the

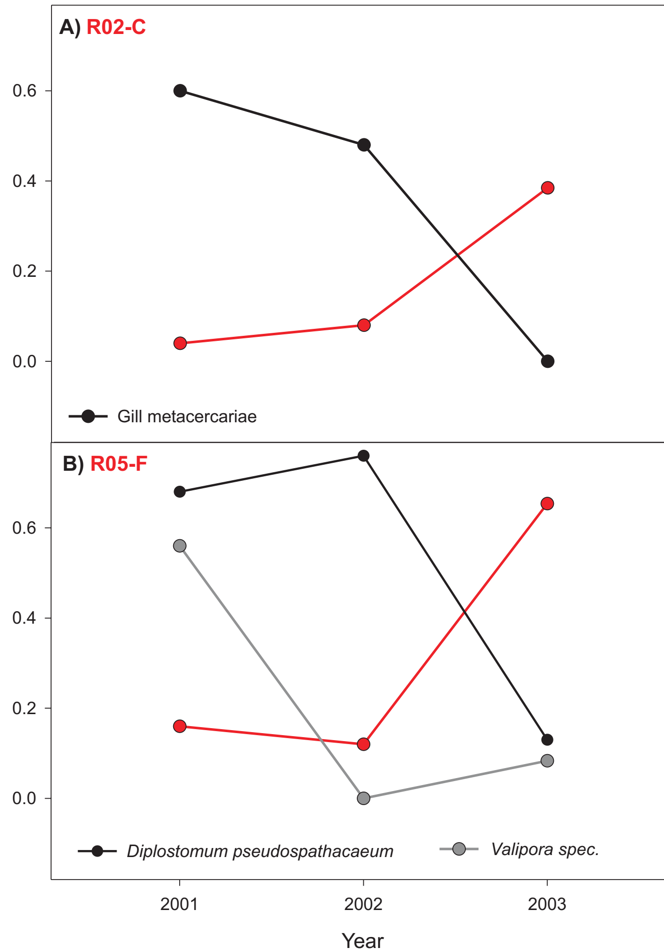


Figure V-5: Time course of MHC class IIB allele frequencies and parasite prevalences, showing significant associations between infection and presence of the allele within individuals in the ditch at Vierer See (VG). A) The red line shows the frequency of MHC class IIB allele R02-C, which is significantly associated to infection with gill metacercariae (black line). B) The red line shows the frequency of MHC class IIB allele R05-F, which is significantly associated with resistance to both the cestode *Valipora campylancristrota* (grey line) and the digenean trematode *Diplostomum pseudospathacaeum* (black line).

microsatellite loci we used are linked to a gene of selective importance. Such microsatellite alleles would then hitchhike with genes under selection and would show stronger frequency fluctuations than truly neutral markers. Two out of the three microsatellite alleles with significant fluctuations in two independent populations (VS and SCW) belong to the same locus, i.e. GAC 1097. Futhermore, the allele GAC1097-109 follows the same frequency pattern as the MCH class IIB allele R07b in SCW and is therefore also associated to the prevalence of the *Raphidascaris acus* cysts in the liver (Fig. V-4). We do not believe that GAC 1097 itself is linked to the MHC class II region, because correlation between alleles of GAC 1097 and MHC class IIB is not significantly different from correlation between MHC class IIB and all other microsatellite loci (mean Spearman's rank correlation for GAC 1097 alleles 0.004, mean Spearman's rank correlation for other microsatellite loci 0.006, ANOVA $F = 0.2937$, $P = 0.7455$). Nevertheless, we cannot exclude the possibility that GAC 1097 is linked yet to another gene outside the MHC, which is associated with nematode infections. Notwithstanding, stronger differentiation at MHC class IIB genes compared to microsatellites, along with the observed coupling of fluctuations in frequency of MHC class IIB alleles (Fig. V-1, Tab. V-1) to the prevalence of parasites (Fig. V-4,5, Tab. V-2) suggests that selection by parasites is causing the observed genetic differentiation in addition to drift.

The parasite fauna between the sites SCW and VG/VS is substantially different (Kalbe et al. 2002). Therefore, it is not surprising that different parasite species fluctuate and different alleles are associated to these parasites (Fig. V-4,5, Tab. V-2). Differences in parasite fauna between VG and VS are less pronounced and there was considerable overlap in species with significant changes in prevalence (Tab. V-2). Additionally, the only allele with significant fluctuations in VS (i.e. R05-F) is also fluctuating in VG, where the allele is conferring resistance to infection from *Valipora campylancristrota* and *Diplostomum pseudospathacaeum*. Why then did we not find similar associations in VS despite the strong associations in fish from VG? One possible explanation might be that genotypic diversity of fish hosts as well as that of parasites is larger within VS. At least for fish hosts this assumption is met, because effective population size is nearly 3 times larger in VS than in VG. We do not have similar N_e estimates for parasites. However, we would expect that first intermediate hosts of stickleback parasites will be less common in the small tributary VG, leading to relatively fewer parasite genotypes, with a concomitant decrease in N_e. Strong associations might be easier to detect when

diversity of matching alleles is smaller. When different parasite genotypes are associated with different MHC alleles, higher parasite genotype diversity will blur any association as long as infection by a parasite species as a whole cannot be attributed to different parasite genotypes.

The MHC class IIB alleles conferring resistance, in particular, show properties of Red-Queen dynamics. Here, rare alleles rise with simultaneous decrease of parasite prevalence, and vice versa (Fig. V-4,5). However, this study is based on data from only three generations of fish, which is probably insufficient to show the recurrent cycling of host and parasite genotypes predicted. If host MHC genotypes and parasites cycle antagonistically, clear predictions can be derived for future generations in these populations. Nevertheless, three generations were sufficient to discover significant changes in frequency in MHC alleles in passerines (Westerdahl et al. 2004a) and to show the first part of the predicted oscillations by the Red-Queen theory in this study (Fig. V-4,5). This is by far more dynamical than predicted by theoretical models, where full oscillations take ≈30 generations in simple matching allele models (Peters and Lively 1999; Agrawal and Lively 2002). The speed of fluctuations shown here supports the role of parasites as one of the prime co-evolutionary forces explaining the extraordinary polymorphism of MHC genes by both overdominance and negative frequency dependent selection.

Conclusion

The tremendous variation of MHC genes can obviously not be neutral in the face of natural selection (Clarke and Kirby 1966). For example, at the most polymorphic loci of the human MHC, the HLA-*B* locus and the HLA-*DRB1* locus, 563 alleles respectively 447 alleles can be found (Robinson et al. 2003). It is hard to envisage that the extremely high number of alleles is not selectively maintained. Balancing selection, operating by overdominant or negative frequency dependent selection, mediated by pathogens is the most parsimonious explanation for the maintenance of polymorphism (Apanius et al. 1997; Penn and Potts 1999). Several studies have demonstrated the contribution of overdominance by showing that MHC heterozygotes are able to detect a wider range of pathogens (Doherty and Zinkernagel 1975) and are therefore more resistant to infection (Nevo and Beiles 1992; Thursz et al. 1995; Carrington et al. 1999; Senseney et al. 2000; Hedrick et al. 2001a; Arkush et al. 2002; Grimholt et al. 2003). But not all studies, inculding work from this thesis, find support for overdominant selection (Wegner et al. in press). In simultaneous multi-pathogen challenges MHC heterozygotes seem to have a general advantage (Penn et al. 2002; McClelland et al. 2003; Wegner et al. 2003a, Chapter II; Wegner et al. 2003b, Chapter I; Kurtz et al. 2004). However, following theoretical predictions (Nowak et al. 1992; Borghans et al. 2003) maximal heterozygosity at MHC loci is also detrimental, because negative thymic selection reduces the diversity of naïve T cells, when MHC diversity is too high. In three-spined sticklebacks *Gasterosteus aculeatus* intermediate levels of individual MHC diversity are indeed associated with lowest parasite burden (Wegner et al, 2003b, Chapter I, Wegner et al. 2003a, Chapter II, Chapter IV). Furthermore lower parasite burden can lead to increased survival of fish with intermediate MHC diversity under severe environmental stress (Chapter IV). This shows that an optimal MHC diversity, presumably correlated with superior Darwinian fitness, exists within natural ranges of individual MHC diversity in sticklebacks. So far, evidence for detrimental effects of increased individual MHC diversity was less direct and mainly stems from polyploid populations, which seem to suppress expression of their surplus copies of MHC genes (Du Pasquier et al. 1989; Dixon et al. 1996; vanErp et al. 1996; Kruiswijk et al. 2004). The mechanistic route of expression regulation of MHC genes is also taken by sticklebacks, because MHC class IIB expression levels correlate negatively to MHC diversity in individuals

and families (Chapter III). For these reasons it becomes obvious that despite increased immunological surveillance (Doherty and Zinkernagel 1975) further expansion of the MHC class II region by additional duplication of loci will face counter-selection. Furthermore, selection for MHC optimization must have been strong enough to let the highly sophisticated MHC mediated mate choice mechanism of sticklebacks evolve. Here, females try to complement their own set of MHC alleles with those of the male to reach a combined diversity that maximizes the offspring's chance to end up with an optimal number of alleles (Reusch et al. 2001a; Aeschlimann et al. 2003). Therefore, sticklebacks form a unique example for congruent aims of natural and sexual selection acting on polymorphic MHC genes by parasites.

Fewer studies tried to evaluate the role of negative frequency dependent selection for the maintenance of genetic variability of MHC genes. In fact, only a single study found significant changes in MHC allele frequencies between generations (Westerdahl et al. 2004a). Nonetheless, the fluctuations of host genotypes being tracked by parasites remains a crucial prediction of the Red-Queen hypothesis of antagonistic co-evolution (Van Valen 1973). In three populations of sticklebacks we found an excess of fluctuating MHC alleles compared to neutral microsatellite loci within three generations. Since some of these alleles were associated to resistance and infection by single parasite species, correlative evidence for parts of the predicted negative frequency dependence were found (Chapter V). This indicates that both mechanisms of balancing selection, overdominance and negative frequency dependent selection, act to maintain the extreme polymorphism of stickleback MHC genes.

Sticklebacks are becoming an increasingly important model organism for evolutionary biology (Schluter 1994; Rundle et al. 2000; Peichel et al. 2001; Reusch et al. 2001a; Cresko et al. 2004; Shapiro et al. 2004). The repeated successful colonization of habitats with subsequent occurrence of large natural populations turn sticklebacks into ideal candidates to investigate natural selection in near field situations. The ease of breeding and maintaining fish in the laboratory makes an experimental approach also feasible. Biotic interactions leading to co-evolution like symbiosis or parasitism have, however, been rarely addressed by experimental studies. Here, we elucidated the role of MHC class IIB genes as a major quantitative trait locus controlling infection by pathogens, but also behaviour in terms of mate choice (Reusch et al. 2001a). However, next to MHC genes a lot of other genetic factors are likely to be subject of the arms race between sticklebacks and their parasites. Especially, the trade-off between the two arms of the

immune system – innate and adaptive immunity - deserves further attention (Kurtz et al. 2004). Genes associated with the innate immune response are likely to eliminate the vast majority of infective pathogens (Janeway and Medzhitov 2002) and identification of heritable differences within these genes – may they be regulatory or structural- will considerably advance the knowledge of immunity. The development of genetic tools for sticklebacks (linkage mapping (Peichel et al. 2001), expression studies (Chapter III) and genome sequencing) opens up the possibility to identify these genes and get a much more detailed picture of the interaction between parasites and their hosts in a natural environment.

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Glossary

Term	Definition
Alleles	Different forms of a gene found at the same locus, assumed to arise by mutation
Balancing selection	Form of selection, which favours more than one variant; operates mainly by overdominance or negative frequency dependent selection
DNA	Deoxyribonucleic acid, the genetic material of all cells and many viruses
Gene	Set of DNA sequences that are required to produce a single polypeptide; "Unit of inheritance"
Genetic drift	Random change in allele frequency within a population
Genetic locus	The position of a gene on a chromosome
Heterozygote	Nucleus, cell or organism with different alleles of one or more specific genes
Homozygote	Nucleus, cell or organism with identical alleles of one or more specific genes
Individual MHC diversity	The number of different MHC alleles within an individual; due to duplications of MHC loci more than two can be present in an individual
Molecular markers	Identifiable physical locations on a chromosome
MHC	Major histocompatibility complex, gene cluster in vertebrates mainly associated with immune function; Genes products of class I and class II present bound peptides to T cells
Microsatellite	Short sequences of di- to tetranucleotide repeats of very variable length. Using PCR primers to the unique sequences upstream and downstream of a microsatellite their location and polymorphism can be determined; supposingly selectively neutral
Mutation	Change in DNA sequence of an organism
Negative frequency dependent selection	Rare allele advantage, the fitness contributed by an alleles is negatively correlated to its frequency in the population
Overdominance	Heterozygotes have a fitness advantage over both homozygotes
PCR Polymerase chain reaction.	The first practical step for *in vitro* amplification of DNA.
Reverse Transcription (RT)	Transcription from RNA to DNA, RNA-Viruses integrate into the host genome by reverse transcription; used in vitro to transcribe labile mRNA to more stabile cDNA
Red Queen hypothesis	The rapid dynamics of antagonistic co-evolution are described by this metaphor from Lewis Caroll's book "Through the looking glass"
RNA	Ribonucleic acid, used a messenger RNA (mRNA) for transcription in the translation process from nuclear DNA to proteins

Appendix

The Science Rebuttal

Publication of Chapter II in Volume 301 of *Science* resulted in a scientific discussion on whether populations should be monomorph with respect to individual MHC diversity.

TECHNICAL COMMENT

Comment on "Parasite Selection for Immunogenetic Optimality"

Resistance to pathogens has been measured directly in several organisms by examining single-copy major histocompatibility complex (MHC) genes (*1, 2*). However, some MHC genes are found in multiple, tightly linked copies, as expected from gene duplication. Wegner *et al.* (*3*) reported that for a class IIB MHC gene with a variable number of copies (three to nine copies in different individuals) in the three-spined stickleback, individuals with an intermediate number of alleles (gene copies) appear to have the lowest parasite load in experimental immune challenges.

To understand how such selection would influence genetic variation, I used a model in which an intermediate optimum is favored, similar to the quadratic deviations model (*4, 5*). Here, the fitness of the genotype with i alleles is $w_i = 1 - k(P_i - P_o)^2$, where P_i and P_o are the phenotypes (number of alleles) for phenotype i and the optimum phenotype, respectively, and k is a constant determining the amount of selection. As assumed by Wegner *et al.* in (*3*), I assumed that all alleles were qualitatively similar—that is, all individuals with a given number of alleles had the same fitness, regardless of the identity of the alleles.

Let us assume that the maximum fitness is for the phenotype with 5.82 alleles, which was the estimated minimal parasite load in (*3*), and assume that there are haplotypes with from one to five alleles. If we assume that $k = 0.01$, then the fitness ranges from about 0.9 for a phenotype with either three or nine alleles to 1.0 for a phenotype with six alleles. With this model, selection results in a monomorphic population consisting of only haplotypes with three alleles. This theoretical result is consistent with that found in earlier research (*4, 5*) in which selection for an intermediate optimum does not generally maintain polymorphism and results in fixation of the haplotype that gives only phenotypes with the maximum fitness.

This finding is in contrast to the distribution of haplotypes observed by Wegner *et al.* (*3*), in which the whole distribution of phenotypes from three to nine alleles was observed in 86 individuals (see Fig. 1) and the phenotypic diversity is $1 - \Sigma x_i^2 = 0.822$, where x_i is the frequency of the ith phenotype. One way to counter the effect of selection reducing variation in the number of alleles is to incorporate mutation to chromosomes with different numbers of alleles (unequal crossing over to produce duplications or deficiencies). Let us assume that u is the rate of mutation from a haplotype with i alleles to a haplotypes with $i + 1$ alleles and to $i - 1$ alleles (a proportion $1 - 2u$ do not mutate). If $u = 0.05$, then the equilibrium distribution is as given in Fig. 1. This is similar to that observed and has a similar phenotypic diversity of 0.849. However, this rate of mutation is several orders of magnitude higher than observed for gene duplication and deficiency (*6*), so it seems unlikely that it is the basis of the distribution.

Perhaps a more likely scenario is that the impact of stabilizing selection is even smaller than assumed here, which may make it of little influence in a finite population, and that nonselective forces primarily influence the distribution of allele number. Another possible explanation for the variation in allele numbers is that diversifying selection, either in space or time (*2, 7*), may maintain different alleles at a given locus. However, it is not clear how this could maintain different numbers of alleles within a population.

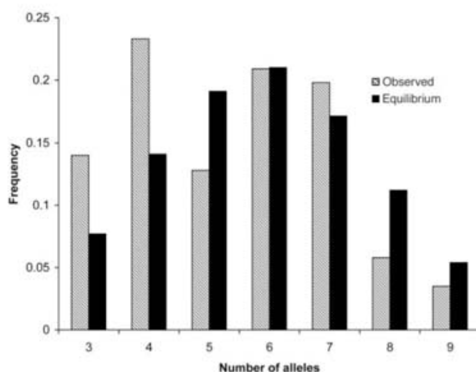

Fig. 1. The observed distribution in three-spined sticklebacks of MHC genotypes with different numbers of alleles from Wegner et al. (3) (hatched bars) and that expected when there is an equilibrium between selection favoring an intermediate optimum and mutation at a rate u = 0.05 to new numbers of alleles (solid bars).

Philip W. Hedrick
School of Life Sciences
Arizona State University
Tempe, AZ 85287, USA
E-mail: philip.hedrick@asu.edu

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TECHNICAL COMMENT

Response to Comment on "Parasite Selection for Immunogenetic Optimality"

The superior parasite resistance of stickleback major histocompatibility complex (MHC) genotypes with an intermediate number of about six alleles has been demonstrated under both natural (*1*) and experimental conditions (*2, 3*). The current debate does not concern the validity of these findings, but rather the machinery of evolution and population genetics maintaining or eliminating the observed variation around the optimum.

Hedrick (*4*) used a simple model to show that optimizing selection should result in a monomorphic population with respect to allele numbers. However, a stable monomorphic population can exist only under unrealistic assumptions implicit in the model. It could consist of individuals all carrying totally distinct haplotypes (Fig. 1A), which requires an infinite number of alleles. Otherwise, as soon as two gametic haplotypes share alleles, recombination leads to offspring with a suboptimal number of alleles. Alternatively, monomorphic populations might be maintained when all genotypes are identical (i.e., six homozygous loci, each bearing a different allele; Fig. 1B). Under this scenario, the six different alleles must be adapted to the local parasite fauna by associations with pathogens (*5*). This would be possible, but only if parasite pressure remains constant. On the other hand, given the high number of alleles in stickleback populations, haplotypes are obviously not fixed (*1*).

An explicit assumption in Hedrick's model (*4*) is that all alleles confer similar resistance toward infection. Because in reality pathogens vary spatially and temporally (in influenza pandemics, for example), the quality of alleles is essential for predicting individual fitness. Such "Red Queen" dynamics are thought to maintain the typical polymorphism of MHC genes found in sticklebacks (*3, 6, 7*) and other vertebrates (*8*). The time lag generated by host-parasite coevolution is likely to create an array of suboptimal genotypes. Furthermore, in multi-locus systems such as stickleback MHC, polymorphism in allele number will be maintained by linkage disequilibrium (LD) for extended periods of time, as pointed out by Lewontin in (*9*). LD will be constantly renewed by parasite selection because different parasites will select for haplotypes carrying specific MHC alleles that confer resistance (*5*)—even if the total number of alleles they bear is deviating from the optimum (i.e., trade-off between allele quality and optimal number of alleles). LD will also create a reservoir of alleles associated with resistance against diseases that temporarily disappeared from the population. Reoccurring parasites will therefore not require new mutations (*10*).

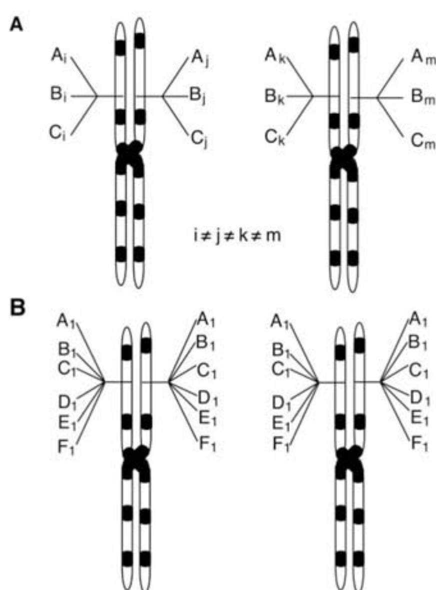

Fig. 1. Theoretical parental genotypes depicting conditions that would have to be met to guarantee a stable, monomorphic population of optimal genotypes. (A) All haplotypes consist of three loci (A, B, and C), which are completely distinct with respect to the alleles they bear. This requires an infinite number of alleles. (B) All individuals are identical and homozygous for their six loci (A through F).

As soon as one accepts that multiple MHC alleles can be selectively maintained within populations, one also buys its consequences for the individuals. Under random mating, the degree of polymorphism at each locus dictates an individual's number of alleles. In order to escape the stochastic nature of Mendelian inheritance (i.e., segregational load), there is selection on females to optimize offspring MHC diversity with reference to their own MHC genotype (*7, 11*). Since females can potentially perceive only genotypes, and not haplotypes, their mate choice will be imperfect. For example, the mating between two optimal genotypes with six alleles each might result in suboptimal genotypes when the haplotypes of each parent consist of packages (gametes) of two and four different alleles. The expected distribution of filial genotypes would then result in only 50% of genotypes with the optimal number of six alleles. In this sense, the Mendelian mixing of haplotypes can be regarded as a constraint of sexual recombination in diploids. It will inevitably create a population of individuals that differ in their number of MHC alleles. LD and Mendelian inheritance only maintain individual variation in the number of MHC alleles if allelic variation on the population level is maintained by ever changing parasites. We have thus shown that a number of basic population genetic processes will always generate variance in allele numbers. These processes should be considered in future models.

K. Mathias Wegner
Martin Kalbe
Joachim Kurtz
Thorsten B. H. Reusch
Manfred Milinski
Max-Planck-Institute of Limnology
Department of Evolutionary Ecology
August-Thienemann-Str. 2
24306 Plön, Germany
E-mail: wegner@mpil-ploen.mpg.de

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CURRICULUM VITAE

Name:	Karl Mathias Wegner
Geburtsdatum:	05.03.1974
Geburtsort:	Niebüll, Nordfriesland

SCHULAUSBILDUNG

1980 - 1981	Grundschule, Tinnum
1981 - 1984	Norddörfer Grundschule, Wenningstedt
1984 - 1993	Gymnasium Sylt, Westerland Allgemeine Hochschulreife (Note 1,9)

ZIVILDIENST

07/1993 - 10/1994	Zivildienst bei der Naturschutzgemeinschaft Sylt, Braderup

STUDIUM

10/1995 - 02/1998	Grundstudium der Biologie an der Georg-August-Universität, Göttingen (5 Semester)
10/1997	**Vordiplom** (Note: “Sehr gut”)
02/1998 - 01/1999	Auslandsjahr an der University of Otago, Dunedin, Neuseeland (gefördert durch den DAAD) **Abschluß**: Postgraduate Diploma in Science in Zoology (DipSci Zoology, Award with distinction)
03/1999- 02/2001	Fortsetzung des Hauptstudiums an der Georg-August-Universität, Göttingen: **Fächer:** • Zoologie (Hauptfach) • Humangenetik • Informatik
07/2000 – 08/2001	**Diplomarbeit** am Max-Planck-Institut für Limnologie Plön,
02/2001	**Diplomprüfung** (Note: “Sehr gut”)
Seit 10/2001	**Promotion** am Max-Planck-Institut für Limnologie Plön

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- Reusch, T. B. H., H. Schaschl and K. M. Wegner (in press). "Recent duplication and inter-locus gene conversion in major histocompatibility class II-genes in a teleost, the three-spined stickleback." **Immunogenetics**.

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Erklärung

Hiermit versichere ich, dass diese Abhandlung – abgesehen von der Beratung durch meine akademischen Lehrer – nach Inhalt und Form meine eigene Arbeit ist, und dass ich keine anderen als die angegebenen Hilfsmittel und Quellen verwendet habe. Die Arbeit hat bisher weder ganz noch zum Teil an anderer Stelle im Rahmen eines Prüfungsverfahrens vorgelegen. Teile dieser Arbeit wurden als Manuskripte bei Zeitschriften eingereicht und veröffentlicht: Kapitel I wurde mit Martin Kalbe und Thorsten Reusch als Koautoren im *Journal of Evolutionary Biology* veröffentlicht, Kapitel II wurde mit Martin Kalbe, Joachim Kurtz, Thorsten Reusch und Manfred Milinski als Koautoren in *Science* veröffentlicht. Teile der Einleitung wurden in Form eines Reviews mit Martin Kalbe, Helmut Schaschl und Thorsten Reusch in *Microbes and Infections* veröffentlicht.

Plön, den 20. September 2004

Mathias Wegner