
**The role of the pleiotropic genes of the major
histocompatibility complex in evolution:
The example of the three-spined stickleback**

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Der Dekan

“... in Science you have to be
frustration resistant...”

A senior scientist observing my
frustration during project development

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Summary

Plants and animals share their environments with a rich fauna of parasites and potentially, there is no species without at least one pathogen. Selection by parasites is recognized as one of the principal evolutionary processes and has probably even led to the evolution of sexual reproduction- a major paradox in evolutionary biology.

To counter this constant threat, vertebrates have developed different immune defenses. In particular the major histocompatibility complex (MHC) plays an important role in the vertebrate immune system. MHC molecules present self and non-self peptides to T-cells and when a foreign peptide is bound it triggers an immune reaction. Since a given MHC molecule can only present a limited number of different peptides, an immune response can be initiated against a limited amount of pathogens per MHC allele. Probably due to this limitation and the immense diversity of parasites, MHC genes present the highest degree of polymorphism reported in jawed vertebrates. This extreme intra- and inter-individual polymorphism is thought to be under balancing selection. The mechanisms by which MHC polymorphism could be maintained are fiercely debated and several theories have been proposed: heterozygote advantage, negative frequency dependent selection and/or habitat heterogeneity.

In addition to its puzzling polymorphism, females have been shown to exert MHC-dependent mate choice. Such preference may be better understood in the light of commonly cited indirect benefits of mate choice including the search for good genes, inbreeding avoidance and acquisition of resistance genes for the progeny.

In the work detailed in this thesis, I (and my co-authors) used the three-spined stickleback as a model organism to investigate the role of the MHC in evolution. In an initial study (Chapter I), we explored whether MHC genes were correlated to important individual fitness traits. While we found a higher nest quality for males harboring an optimal MHC class II*B* diversity, male breeding coloration was most intense at a maximal MHC class I diversity. Two MHC class I alleles were identified to be associated with a higher intensity of red coloration. In this study, we showed that individual sexual displays are MHC-dependent and thus could influence female mating decision.

It had previously been demonstrated that fish with an intermediate MHC diversity were better at resisting parasites. Our results from chapter I showed that the MHC affects sexual characters as well. However, the ultimate measure of evolutionary fitness is the reproductive success. We combined this knowledge in a field experiment to test the relation between MHC and reproductive success- the ultimate factor of evolution (Chapter II). Using laboratory-bred families segregating for MHC genes, we found that reproductive success was higher at

intermediate MHC diversity. In order to test for the generality of this phenomenon, we stocked the same field enclosures with wild-caught fish (Chapter III). We confirmed that with reference to their own MHC profile, female sticklebacks preferred to mate with males sharing an intermediate MHC diversity. Moreover, males with a specific MHC haplotype were better at resisting a common parasite (*Gyrodactylus* sp.) and were more likely to be selected by females. Interestingly, this translated directly into Darwinian fitness since fish harboring this specific MHC haplotype had a higher reproductive output. We concluded that females based their mating decision on a specific MHC haplotype conferring resistance against a common parasite. With this strategy, females achieve a two fold immunogenetic advantage: resistance against a wide range of parasites while keeping the T-Cell repertoire depletion to a minimum level and resistance against a currently common and costly parasite. The latter can be described as “good genes”. Such an interaction between host and parasite driving assortative mating is a pre-requisite to negative-frequency-dependent selection- a potential mechanism to explain MHC polymorphism. Our study not only identified MHC as good genes but also suggested that females might adopt reproductive strategies that minimize disruption of locally adapted MHC genes against parasites.

Such results motivated a reflection on the role of host MHC and parasite interaction in the evolution of population divergence. In chapter IV, we proposed that MHC genes could play a pleiotropic role in parasite resistance and female mating strategy. Individual MHC diversity is restricted compared to the entire allele pool due to a low optimal number of MHC loci. In contrasting niches, parasites communities differ and thus the pools of MHC alleles also differ between niches since not all alleles can be exhibited. Mate choice for the best adapted MHC genotype will favor local adaptations and will increase the pace of population differentiation and speciation. We therefore propose to consider MHC genes as potential “magic traits”.

To investigate the pleiotropic role of MHC genes, we sampled stickleback populations from a lake and a connected river (chapter V), which previous studies have suggested represent ecomorphs. The two fish populations harbored different parasite communities. Most probably as a result of parasite pressure, we found substantial differences in MHC allele pools between the two ecomorphs. If our predictions are correct, we would expect females to exhibit assortative mating for males harboring locally adapted MHC alleles. We performed mate choice experiment based on the genetic component of odor cues, and found that female preference was directed towards males originating from the same habitat. Again, this suggests a pleiotropic role of MHC genes through both parasite resistance and mate choice and reinforces the idea of “magic traits”. Despite a range of observational evidence, no direct experimental study to date has revealed the connection of locally adapted MHC genes and local parasites in a natural habitat.

We decided to challenge this question (Chapter VI) and for this test, we crossed three-spined sticklebacks from the same river and the same lake population as those used in the previous chapter. The populations displayed almost totally distinct MHC allele pools. Intercrossing F1 hybrids, we obtained a second fish generation segregating into pure river, pure lake and two hybrid MHC genotypes, while at the same time the genetic background was randomized. We used a double common garden experiment where fish were exposed simultaneously and reciprocally into the lake and into the river using replicated cages. We found that MHC genotypes were not equal in resisting parasites, which resulted in differences in parasite load between genotypes in turn. The total parasite load harbored by the fish appeared to result from an adaptive interaction between the innate and the acquired immune system of the hosts, which were driven by different parasite strategies. Generalist parasites were more successful in river fish lines, which naturally encounter low infection pressure from these parasites. However, when confronted with a stickleback specific parasite, which is commonly present in river habitats, the fish bearing lake MHC genotypes were more infected than fish carrying river MHC genotypes. In addition, we found support for an additive and synergistic role of genetic background and innate immunity in parasite resistance. Thus, this first test supports the adaptive connection between MHC and parasites and reinforces our previous suggestion of the pleiotropic role of MHC genes.

Eventually, in the last chapter (VII), I report the initial results of another experimental test. We aimed at testing the potential role of negative frequency dependent selection in maintaining MHC polymorphism. Our design not only included selection by parasites but also included female mate choice. We created 6-replicated populations, each formed by 6 different laboratory-bred families in equal proportions and sex ratio. Three experimental populations were exposed to *Camallanus lacustris*, whilst the remaining three were confronted with *Anguillicoloides crassus*. These parasites were selected to be present in the original population where the founders of the lab-bred populations were caught. Letting the system evolve, we assumed that females would mate with partners that would possess a resistant allele against the experimental parasite, and thus increase the frequency of this allele in the following generation. Eggs were collected and raised in the laboratory, and the new stickleback generation was also exposed to both parasites. We hypothesize that fish from parents that faced one parasite, e.g. *Camallanus lacustris*, will be less heavily infected by this parasite than those derived from parents that encountered *Anguillicoloides crassus* and vice versa. As this experiment is running over several stickleback generations, the final dissection of the F2 fish has not yet taken place. Hence, I report the different steps that have been performed to date.

Zusammenfassung

This summary has been translated from English into German by Tobias L. Lenz.

Alle Pflanzen und Tiere sind von einer reichen Parasitenfauna umgeben und es gibt möglicherweise keinen Organismus, der völlig frei von Pathogenen ist. Selektion durch Parasiten ist einer der wichtigsten evolutionären Prozesse und hat vermutlich sogar zur Entwicklung der sexuellen Fortpflanzung, einem der größten Paradoxa der Evolutionsbiologie, geführt.

Wirbeltiere haben ein komplexes System an Immunantworten entwickelt, um dieser konstanten Bedrohung zu begegnen. Eine besondere Rolle spielt hier der Haupthistokompatibilitätskomplex (MHC). MHC Moleküle präsentieren körpereigene und körperfremde Peptide auf der Zelloberfläche, die von T-Zellen erfasst werden und im Falle von körperfremden Peptiden eine Immunreaktion auslösen. Da ein einzelnes MHC Molekül nur eine beschränkte Anzahl von verschiedenen Peptiden binden und präsentieren kann, wird entsprechend pro MHC Allel auch nur gegen eine begrenzte Zahl von Pathogenen eine Immunantwort eingeleitet. Vermutlich aufgrund dieser Einschränkung und der gleichzeitig unermesslichen Parasitendiversität, weisen MHC Gene die höchste bisher unter kiefertragenden Wirbeltieren bekannte Variabilität auf. Dieser extreme intra- und interindividuelle Polymorphismus ist vermutlich unter balancierender Selektion entstanden. Die Mechanismen, durch die diese MHC Variabilität aufrechterhalten wird, unterliegen einer anhaltenden Diskussion und mehrere Theorien sind vorgeschlagen worden: Heterozygotievorteil, negative frequenzabhängige Selektion und/oder Lebensraumheterogenität.

Zusätzlich zu seiner außerordentlichen Variabilität wird dem MHC auch eine wichtige Rolle bei der weiblichen Partnerwahl zugeschrieben. Diese Funktion wird besser verständlich im Zusammenhang mit häufig zitierten indirekten Fitnessvorteilen der Partnerwahl, wie der Suche nach ‚Guten Genen‘, Inzuchtvermeidung und die Erlangung von Widerstandsgenen für die Nachkommen.

In meiner Dissertation benutzte ich den Dreistachligen Stichling als Modellorganismus, um die Rolle des MHC in der Evolution zu erforschen. In einer ersten Studie (Kapitel I) erforschten wir, ob MHC Gene mit essentiellen individuellen Fitnessmerkmalen in Verbindung stehen. Während wir eine höhere Nestqualität bei Männchen mit einer optimalen MHC-Klasse II-Diversität fanden, war die männliche Brutfärbung bei maximaler MHC-Klasse I-Diversität am intensivsten. Zusätzlich konnten zwei spezifische Allele der MHC Klasse I ebenfalls mit einer höheren Intensität der Rofärbung in Zusammenhang gebracht werden. In dieser Studie zeigten wir, dass einzelne sexuelle Merkmale MHC-abhängig sind und einen Einfluss auf die weibliche Partnerwahl haben.

In früheren Studien ist demonstriert worden, dass Individuen mit einer mittleren MHC-Diversität besser bei der Abwehr von Parasiten sind. Unsere Resultate aus Kapitel I zeigen, dass der MHC zusätzlich auch sexuelle Merkmale beeinflusst. Was jedoch letztendlich im Verlaufe der Evolution wirklich zählt, ist der Fortpflanzungserfolg. Wir vereinten deshalb die gewonnenen Erkenntnisse in einem Feldexperiment, um die Relation zwischen MHC und Fortpflanzungserfolg direkt zu testen (Kapitel II). Unter Verwendung von Labor-gezüchteten Fischfamilien, die sich in der Aufspaltung ihrer MHC Allele unterschieden, fanden wir, dass der Fortpflanzungserfolg bei mittlerer MHC-Diversität am höchsten war. Wir besetzten die selben Freilandkäfige mit wild gefangenen Stichlingen (Kapitel III), um die Allgemeingültigkeit dieser Beobachtung zu prüfen. Wir konnten bestätigen, dass weibliche Stichlinge solche Männchen bevorzugten, die unter Bezug auf ihr eigenes MHC-Profil eine mittlere MHC-Verschiedenartigkeit aufwiesen. Außerdem waren Männchen mit einem spezifischen MHC Haplotyp am erfolgreichsten im Bekämpfen eines prävalenten Parasiten (*Gyrodactylus* sp.) und besaßen eine erhöhte Wahrscheinlichkeit, von Weibchen gewählt zu werden. Interessantweise führte dies direkt zu erhöhter darwinistischer Fitness, da Fische mit diesem speziellen Haplotypen einen höheren Fortpflanzungserfolg aufwiesen. Wir schlossen daraus, dass Weibchen ihre Partnerwahl auf einen speziellen MHC Haplotypen abstimmten, der erhöhte Resistenz gegen einen häufigen Parasiten verleiht. Mit dieser Strategie erzielten Weibchen einen doppelten immunogenetischen Vorteil: Resistenz gegen eine große Auswahl an Parasiten, bei gleichzeitigem Minimieren der negativen T-Zell-Selektion und Widerstand gegen einen zur Zeit prävalenten und schädigenden Parasiten. Die letzteren können als „gute Gene“ beschrieben werden. Solch eine Interaktion zwischen Wirt und Parasit, die zu selektivem Verpaaren führt, ist eine Vorbedingung für die negative frequenzabhängige Selektion - ein möglicher Mechanismus, um die hohe MHC-Variabilität zu erklären. Unser Ergebnisse identifizieren den MHC nicht nur als Beispiel für ‚Gute Gene‘, sondern lassen auch vermuten, dass Weibchen eine Reproduktionsstrategie wählen, die die Aufspaltung von an lokale Parasiten angepasste MHC Allele minimiert.

Die vorgenannten Erkenntnisse motivierten eine Reflexion über die Rolle des MHC und seiner Interaktion mit der lokalen Parasitenfauna im Bezug auf Populationsdifferenzierung. In Kapitel IV schlagen wir vor, dass MHC Gene eine pleiotrope Rolle in Parasitenresistenz und Partnerwahl spielen. Individuelle MHC-Diversität ist im Vergleich zum gesamten Allelpool durch eine niedrige optimale Anzahl von MHC Genen begrenzt. In kontrastierenden Nischen unterscheiden sich Parasitgemeinschaften und folglich unterscheidet sich auch der MHC Allelpool zwischen Nischen, da nicht alle Allele in einem Individuum auftreten können. Partnerwahl für den lokal bestangepassten MHC Genotyp führt somit zu lokaler Anpassungen und erhöht die Geschwindigkeit der Populationsdifferenzierung und Artbildung. Wir schlagen deshalb vor, MHC Gene als potentielle sogenannte „magic traits“ zu sehen.

In Kapitel V beprobten wir Stichlingspopulationen in einem See und einem verbundenen Fluss eines bereits studierten Gewässersystems, um die pleiotrope Rolle der MHC Gene zu erforschen. Die beiden Populationen beherbergten verschiedene Parasitengemeinschaften. Wir fanden erhebliche Unterschiede im Allelpool der beiden Ökotypen, die vermutlich aus divergierendem Parasitendruck resultieren. Wenn unsere Vorhersage aus Kapitel IV korrekt sein sollte, würden wir erwarten, dass Weibchen Partner wählen, die lokal angepasste MHC Allele tragen. Mit Hilfe von olfaktorische basierten Partnerwahlversuchen fanden wir heraus, dass Weibchen solche Partner bevorzugten, die aus dem selben Lebensraum stammten. Dieses Ergebnis bestätigt die pleiotrope Rolle der MHC Gene in Parasitenresistenz und Partnerwahl und bestärkt die Vorstellung als „magic traits“. Neben den verschiedenen empirischen Nachweisen gibt es noch keinen direkten experimentellen Test, der die lokale Anpassung von MHC Genen auf sympatrische Parasiten in einem natürlichen Lebensraum nachweist.

Wir entschieden uns daher, diesen Nachweis anzugehen (Kapitel VI). Hierfür kreuzten wir Dreistachlige Stichlinge aus dem selben Fluss und dem selben See wie die, die im vorhergehenden Kapitel untersucht worden waren. Die Populationen wiesen fast vollständig getrennte MHC Allelpoole auf. Durch das Kreuzen von F1 Hybriden erhielten wir eine zweite Fischgeneration, die sich, was den MHC betraf, in reine Fluss-, reine See- und Hybrid-Genotypen aufspaltete, während der genetische Hintergrund randomisiert wurde. Die Fische wurden in replizierten Käfigen gleichzeitig und reziprok den See- und Flussbedingungen ausgesetzt. Wir fanden heraus, dass MHC Genotypen sich in der Parasitenresistenz unterschieden. Die allgemeine Parasitenbelastung der Fische schien aus einer adaptiven Interaktion zwischen dem angeborenen und erworbenen Immunsystem zu resultieren, wiederum beeinflusst durch verschiedenen Parasitenstrategien. Generalisten unter den Parasiten waren in Fischen mit Flusshintergrund erfolgreicher, die natürlicherweise einem niedrigen Selektionsdruck durch diese Parasiten ausgesetzt sind. Fische mit See-spezifischem MHC Genotyp waren dagegen stärker mit einem stichlingsspezifischen Parasiten, der häufig im Fluss auftritt, infiziert als Fische mit Fluss-spezifischem Genotypen. Zusätzlich fanden wir im Bezug auf Parasitierung Hinweise auf additive und synergistische Effekte zwischen dem genetischen Hintergrund und dem angeborenen Immunsystem. Somit zeigt dieser erste Test die adaptive Verbindung zwischen MHC und Parasiten und bestärkt die von uns vorgeschlagene pleiotrope Rolle der MHC Gene.

Im letzten Kapitel (VII) berichte ich schließlich über die Anfangsschritte eines weiteren experimentellen Tests. Wir streben an, die mögliche Rolle der negativen frequenzabhängigen Selektion im Bezug auf die Erhaltung der MHC-Diversität zu untersuchen. Unser Design beinhaltet nicht nur die Selektion durch Parasiten, sondern auch weibliche Partnerwahl. Wir kombinierten 6 verschiedenen laborgezüchtete Stichlingsfamilien, um 6 replizierte

Populationen zu erhalten, die in 6 identischen Außentanks gehalten wurden. Drei dieser experimentellen Populationen wurden mit *Camallanus lacustris* infiziert, während die drei anderen mit *Anguillicoloides crassus* konfrontiert wurden. Diese Parasiten wurden gewählt, da sie im ursprünglichen Habitat der laborgezüchteten Fische vorkommen. Wir erwarten, dass Weibchen sich eher mit Partnern verpaaren, die ein Allel besitzen, welches Resistenz gegen den experimentellen Parasiten verleiht und sich folglich die Häufigkeit dieses Allels in der folgenden Generation erhöht. Nachdem wir die gesammelten Fischeier im Labor aufgezogen hatten, werden wir diese neue Generation mit den beiden experimentellen Parasiten konfrontierten. Wir erwarten, dass Nachkommen der Eltern, die einem Parasiten, z.B. *Camallanus lacustris*, ausgesetzt waren, weniger schwer durch diesen Parasiten infiziert werden, als die die unter dem Einfluss des anderen Parasiten, also *Anguillicoloides crassus* erzeugt wurden. Dieses Experiment beinhaltete mehrere Fischgenerationen und konnte deshalb innerhalb der normalen Doktorandenzeit nicht abgeschlossen werden. Die Sektion der zweiten Fischgeneration steht noch aus und ich berichte folglich über die verschiedenen Schritte, die bis dato durchgeführt worden sind.

Introduction

I- Host-parasite co-evolution

Understanding the origin of species diversity is the root of evolutionary biology. This question has fascinated generations of naturalists and scientists. Delving deeper into how species form revealed paradoxes, which added to the list of mysteries evolution offers. In evolutionary biology a paradox is a phenomenon, a mechanism or a strategy that appears to be disadvantageous on the short term but is essential for the population on a long time scale, and vice versa. One of the most intriguing paradoxes is the maintenance of sex. Due to higher reproductive success, asexual individuals should predominate, however this is not the case in most taxa. It is now thought that this is linked to the existence of parasites.

In this general introduction after defining parasites and their role in host immune system evolution, I will discuss how parasites affect sexual reproduction- a focus of my PhD work. In the last section of this introduction, I will present the concept of ecological speciation and the potential role parasites can play. This will provide the context for my studies that are reported in the different chapters.

I.1- Parasites

When one organism attempts to exploit another organism as a resource, this can produce selection for change in the exploited organism. In turn, effective defense selects for changes in the exploiter. “The red queen hypothesis” explains how this type of process can prevent the extinction of co-evolving species pairs over evolutionary time scales (Van Valen 1973). Exploiter/victim dynamics characterize many different interactions between organisms: prey/predator, grazed/grazer or host/parasite.

A parasite can be defined as an organism that lives in, or on another living organism, obtaining from it part or all of its organic nutrients, and causing damage to its host (Price 1980). Because this definition is vague, the term parasite has been applied to a wide range of plant and animal taxa. One can even argue that parasites greatly outnumber free-living organisms (Windsor 1998; Kuris et al. 2008) and that there is probably no organism without any parasites (Poulin 1996).

Parasitic exploitation has been the focus of diverse evolutionary hypotheses due to the strong selective pressures it can produce. This results from a strong disparity between host and parasite generation times, providing short-living parasites with advantages (Hamilton 1980; Lively 1999). Organisms have consequently evolved a wide array of genetically coded defenses against parasites to survive and reproduce successfully in any parasite-rich

environment [e.g. plants (Allen et al. 2004), invertebrates (Hoffmann et al. 1999) and vertebrates (Janeway et al. 2001)]. Indeed parasites have been shown to be a source of both sexual (Hamilton & Zuk 1982; Andersson 1994) and natural selection (Poulin 2006) on the host. Despite this continued attention, many aspects of host-parasite interactions remain poorly understood.

Defense mechanisms against parasites can entail behavioral avoidance of parasites or structural defenses, e.g. a thick outer layer. Defense mechanisms at the molecular level also play a central role, typically via host recognition of parasite stimuli. The vertebrate immune system can broadly be divided into innate and acquired responses (Janeway et al. 2001). The innate immune system constitutes a first line of defense against invading parasites and is responsible for inflammation and phagocytosis. It is generally regarded as fast and effective against many pathogens, but is rather unspecific, as it depends on the recognition of conserved molecular patterns shared by large pathogen classes (Dixon & Stet 2001). The adaptive immune defense is highly specific and achieves its extraordinary specificity by somatic rearrangement of genes that encode antigen receptors, i.e. T cell receptors and antibodies (Fearon & Locksley 1996). Genes of the major histocompatibility complex play a key role in this adaptive immune response. It should be noted that the acquired and innate reactions work together - they are tightly linked to each other by many different pathways (Janeway et al. 2001).

1.2- Immune genes

The major histocompatibility complex (MHC; see box. 1) represents the most polymorphic cluster of genes in all jawed vertebrates (Apanius et al. 1997). Several lines of evidence indicate that the high allelic diversity observed at the MHC is maintained through a combination of selective forces. First, where genealogies of MHC allele sequences have been obtained from several species, e.g. primates and felines (Klein et al. 1993; Yuhki & O'Brien 1997), the allelic divergence at MHC lineages predates the speciation event giving rise to separate taxa. This suggests the action of balancing selection over long periods of evolutionary time. Second, comparisons of allelic sequences indicate that the rate of non-synonymous substitutions exceeds the rate of synonymous substitutions at the antigen presenting sites (box2), thus positive selection favors new MHC variants and increases diversity. This can be observed in diverse taxa: e.g. striped mice (Froeschke & Sommer 2005), great snipe (Ekblom et al. 2007), cichlids (Blais et al. 2007), barn owl (Burri et al. 2008), toad (Hauswaldt et al. 2007), horses (Hedrick et al. 1999), primates (Satta et al. 1993), humans (Hughes & Nei 1989). Finally, in humans, the large number of alleles present at MHC loci shows a relatively even distribution across populations, leading to a higher level of heterozygosity

Box 1: MHC molecules activating T cells

There are two main types of MHC molecules, MHC class I and class II. Both MHC molecule classes, called HLA molecules (human leukocyte antigen) in human, function as peptide shuttles that transport peptides from the cytoplasm and display them on the cell surface. The peptides presented by MHC class I molecules are mostly derived from proteins degraded by the proteasome, whereas MHC class II molecules display peptides from extracellular antigens that have been endocytosed and degraded by endosomal and lysosomal proteases. Class I molecules present peptides to CD8+ cytotoxic T lymphocytes and class II molecules to CD4+ T helper cells (Janeway et al. 2001). MHC class I molecules are present on nearly all cell type, while MHC class II molecules are displayed by antigen presenting cells (APCs) such as macrophages, B cells and dendritic cells. The antigen receptors on T cells are specific for complexes of foreign peptides displayed on self MHC molecules. In human, up to six different MHC molecules can be displayed on the cell surface, which are the products of three different MHC class- I genes (A, B and C) in human. These genes are particularly polymorphic in the region that encodes for the peptide-binding domain. Thus, different MHC molecules on APCs bind and display different peptides due to their unique binding motifs, ensuring that a broad variety of antigens can be presented and recognized by T cells. Naive CD8+ T cells, after binding to the MHC-peptide complex on APCs, can develop into cytotoxic T lymphocytes that are capable of lysing peptide-presenting cells. This mechanism allows detecting foreign antigens that are displayed by MHC molecules when cells harbor intracellular infections or extracellular pathogens.

than may be explained under neutral theory and again suggesting the action of balancing selection (Apanius et al. 1997).

Despite strong evidence for balancing selection at the MHC, mechanisms behind maintenance of MHC polymorphism are still unclear. What are the precise selective forces acting on MHC genes? Several studies suggest a role for reproductive mechanisms like MHC-dependent mate choice, selective abortion or genetic incompatibility, as shown in mice and humans (Potts et al. 1991; Wedekind et al. 1995; Wedekind et al. 1996; Tregenza & Wedell 2000). Although this hypothesis might be true, it does not provide an explanation for all vertebrates since they do not all have MHC-driven mating systems. Since the primary function of MHC molecules is to present peptides to T lymphocytes (see box 1), which initiate protection against parasites, the predominant selective forces are likely pathogen-driven.

Box 2: Antigen Binding sites

In this work variation was studied in the most variable part of the MHC Class II B, exon II, which codes for the peptide-binding groove of the MHC molecule.

Antigenic peptides are anchored at specific residues called Antigen Binding Sites (ABS), which are commonly found to be under positive selection. MHC genes that differ at ABS produce molecules that effectively bind and present different ranges of antigens to T cells. This will in turn determine whether or not an effective immune response can be mounted against an infectious agent. The MHC thus initiates the adaptive immune defense.

1.2.1- MHC polymorphism: a consequence of parasite driven selection?

The potential mechanisms by which parasite mediated selection acts on MHC polymorphism can be summarized in three major hypotheses: heterozygote advantage, negative-frequency dependent selection and habitat heterogeneity. It is worth noting that these three mechanisms acting on MHC polymorphism are not mutually exclusive and might work in synergy (e.g. Potts & Slev 1995; e.g. Apanius et al. 1997). This is best seen in the many associations between MHC allelic diversity and resistance to parasitism that have been found in natural populations (e.g. Hill et al. 1991; Langefors et al. 2001; Grimholt et al. 2003; Harf & Sommer 2005; e.g. Bonneaud et al. 2006b) (reviewed in Milinski 2006; Piertney & Oliver 2006) , which could be a sign for heterozygote advantage but also for negative frequency dependent selection.

1.2.1.1- Heterozygote advantage (HA)

It is assumed that the presence of more MHC molecules would enhance the ability to detect and bind a wider array of parasite antigens to subsequently initiate a specific immune response (Doherty & Zinkernagel 1975). The various associations between MHC allelic diversity found in natural populations (see for review Sommer 2005a; see for review Piertney & Oliver 2006) imply that pathogens are detected through one or a few peptides. Therefore it appears advantageous for an individual to be heterozygous at MHC loci.

For decades, evolutionary biologists have been experimentally testing heterozygote advantage under infection challenge. However, the results from these experiments are varied and controversial; some studies support the hypothesis (Doherty & Zinkernagel 1975; Carrington et al. 1999; Penn et al. 2002) while others do not (Paterson et al. 1998; Ilmonen et al. 2007).

In order to maximize MHC heterozygosity, mate choice cannot be random with regard to MHC. Mating strategy should be oriented towards finding the mates that will provide the highest diversity to the offspring- resulting in MHC disassortative mating. Many studies have demonstrated MHC-based female mate choice for disassortative mating, however inbreeding

avoidance is an alternative explanation for this choosiness (e.g Yamazaki et al. 1976; e.g Egid & Brown 1989; Potts et al. 1991; Potts et al. 1994; Olsson et al. 2003).

Although controversial, HA appears plausible. But, is MHC polymorphism maintained by heterozygote advantage? Recently, theoretical work demonstrated that HA alone fails to explain the extreme diversity in MHC (De Boer et al. 2004b). Moreover, stable levels of polymorphism and allele frequencies in MHC, two predictions of HA (Slade & McCallum 1992), have seldom been reported. In summary, HA has not been confirmed unambiguously by a number of studies.

I.2.1.2- Negative-frequency dependent selection (NFDS)

An alternative hypothesis proposes that balancing selection at MHC genes is the result of negative-frequency dependent selection. Under NFDS, a rare allele is more likely to be resistant to common parasite genotypes, and will therefore confer a selective advantage to its bearer. The allele will consequently increase in frequency. However, parasite adaptation to host defenses should lead to selection for a parasite, or parasite strain, more virulent against this common genotype. [Note, virulence is defined as the amount of damage a parasite causes to its host (Ebert & Hamilton 1996).] The host allele will therefore be subject to negative selection and will decrease in frequency as another rare allele gains a selective advantage. This type of selection leads to fluctuations in frequencies of all alleles. Both the fixation and loss of alleles are selected against, thus high allelic diversity is promoted (Clarke & Kirby 1966; Bodmer 1972; Takahata & Nei 1990; Slade & McCallum 1992).

Jawed vertebrates present a reduced optimal number of MHC loci and cannot display the entire potential MHC allele pool present in the populations (Lawlor et al. 1990; Janeway et al. 2001; Robinson et al. 2003). Because of this restriction, MHC alleles present fluctuating frequencies: an allele conferring resistance one year might not have the same advantage the following year and will decrease in frequency.

Although theoretically straightforward, there is little data supporting the negative-frequency dependent selection hypothesis. To my knowledge, only a handful of studies provided hints of a NFDS scenario in MHC. Annual population surveys of great reed warblers (Westerdahl et al. 2004), sticklebacks (Wegner 2004) and soay sheeps (Charbonnel & Pemberton 2005) represent the few examples of significant variation in MHC allele frequencies between successive age cohorts. Parasite-driven selection was invoked to explain these patterns, but female mate choice may also cause fluctuations in allele frequencies.

There are two different ways for an allele to increase in frequency: either the alleles confers a direct selective advantage in the form of parasite resistance, or an indirect advantage because females prefer mates with the allele. For mate choice to follow the predictions of NFDS, females should seek a partner displaying his ability to resist parasitization (Hamilton & Zuk

1982). Resistance to parasites should be MHC driven and detectable by females. A study on great snipes reported evidence for mate choice for males carrying specific MHC alleles (Ekblom et al. 2004), thus, supporting NFDS. However, experimental support for NFDS is awaited.

I.2.1.3- Habitat heterogeneity

A less commonly invoked mechanism promoting MHC polymorphism deals with spatial and temporal variations in the parasite selection regime (Hill 1991). Ecological conditions and community structure of contrasting habitats are likely to influence the presence and abundance of intermediate hosts, which are necessary for the maintenance of some parasites (Thompson 1994). Examples of contrasting terrestrial habitats include forests and open fields, and contrasting aquatic habitats include lakes and rivers. Due to their ecosystem structure, considering biotic and abiotic factors, different habitats are likely to support distinct parasite communities. Various recent studies, investigating MHC variation in neighbouring populations, revealed that habitat heterogeneity shapes MHC composition, probably due to differences in parasite community (Wegner et al. 2003b; Bonneaud et al. 2006b; Blais et al. 2007; Ekblom et al. 2007; Alcaide et al. 2008; Babik et al. 2008). However, often these studies only assumed variation at the parasite community level.

I.1.2.4- Optimality

Although MHC polymorphism has been the focus of numerous studies, there is another striking fact in MHC genes: surprisingly, the intra-individual diversity (defined as the overall number of different classical MHC alleles at all loci) is restricted to a small subset of alleles. Therefore a paradox remains: why doesn't the mechanism that leads to high polymorphism at the population level lead to higher intra-individual MHC diversity? This is even more puzzling given the potential for evolutionary duplication and diversification of individual loci (Lawlor et al. 1990). The T cell repertoire might be at the root of this paradox since its diversity is crucially dependent on the number of MHC molecules, and thus on the number of expressed MHC genes.

In a process called positive selection, T cell receptors are tested for general reactivity to the individual's MHC-molecules. Only T-cells with receptors displaying a certain affinity for binding the complex MHC-molecule/self peptide will be selected. As a result, any increase in individual MHC diversity will increase the number of positively selected T cell lines of the T cell repertoire, enhancing the likelihood of detecting intruding parasites (Doherty & Zinkernagel 1975). After having undergone positive selection, T-cells down-regulate expression of one of the two co-receptors, CD4 and CD8, and develop into either CD4 - single positive or CD8 - single positive T-cells. During negative selection, T-cells, whose receptors react too strongly

with self-peptide – MHC complexes are eliminated. Because self/ non-self discrimination is mediated by MHC molecules, recognition of self peptides as foreign would lead to the activation of the immune system and thus to auto-immune diseases (Germain 1994). Therefore, the balance between positive and negative selection theoretically leads to an optimal number of different MHC molecules (Nowak et al. 1992; Woelfing et al. 2009) or to an optimal MHC heterozygosity (De Boer & Perelson 1993). This prediction was recently confirmed by empirical studies, showing the lowest effect of parasite infections in fish (Wegner et al. 2003a; Wegner et al. 2003b) and reptiles (Madsen & Ujvari 2006) with intermediate MHC diversity.

The optimal number of MHC variants can be obtained in offspring if a female chooses mates with reference to her own MHC make-up. A female with a low number of MHC alleles should seek a male with a high number, where a very heterozygous female should look for a mate offering low MHC diversity (Reusch et al. 2001a; Jacob et al. 2002; Aeschlimann et al. 2003; Olsson et al. 2003; Milinski et al. 2005).

I.2.1.5- MHC based mate choice

There is growing evidence for MHC-dependent mate choice in mice (Yamazaki et al. 1976; Potts et al. 1991; Penn & Potts 1998) birds (Richardson et al. 2005; Bonneaud et al. 2006a), fish (Landry et al. 2001; Reusch et al. 2001a; Aeschlimann et al. 2003; Olsson et al. 2003; Milinski et al. 2005; Forsberg et al. 2007; Consuegra & Garcia de Leaniz 2008), non-human primates (Schwensow et al. 2008) and humans (Wedekind et al. 1995; Jacob et al. 2002) . Why do females make mating decisions based on MHC? Such preferences may be better understood in the light of three commonly cited indirect benefits of mate choice: the search for “good genes” (Hamilton & Zuk 1982), inbreeding avoidance (Yamazaki et al. 1976; Potts et al. 1991) and acquisition of resistance genes for the progeny (Reusch et al. 2001a; Aeschlimann et al. 2003; Milinski et al. 2005; Milinski 2006).

First, “good genes” are genes which increase individual fitness (Andersson 1994). Under this theory, females prefer mating with males displaying more intense secondary sexual traits or any characteristic which is correlated with body condition. Such traits are referred to as honest signals (Zahavi 1975). Body condition is expected to decrease under parasitization (Milinski & Bakker 1990a; Bakker & Mundwiler 1994) and thus might be dependent on MHC genes. Potentially, signals of higher fitness become honest signals which are translated from good genes.

Several studies have shown that MHC signals in odors could reflect the degree of relatedness between individuals and that MHC-based mate choice may reflect the avoidance of incestuous mating rather than the search for good genes (Yamazaki et al. 1976; Potts et al. 1991; Brown & Eklund 1994). On the other hand, using olfactory cues female sticklebacks prefer males with

a complementary number of MHC class II alleles to their own set of genes, resulting on average in intermediate immunogenetic diversity of their offspring (Reusch et al. 2001a; Aeschlimann et al. 2003; Milinski et al. 2005). A female may therefore benefit from mating with a particular partner if she acquires resistance genes for her progeny. Note, in sticklebacks and probably in humans (Gilbert et al. 1986; Jacob et al. 2002) and mice (Yamazaki et al. 1976; Singh et al. 1987), females can assess their potential partners' MHC make-up directly via perception of corresponding peptide ligands in odor signal (Milinski et al. 2005).

II- Parasites and the evolution of sexual reproduction

Host-parasite interactions are likely an important factor promoting the persistence of sexual reproduction as a mechanism to increase genetic diversity via recombination. All else being equal, asexually reproducing organisms should have a substantial reproductive advantage (Trivers 1985). Indeed, the existence of males that do not produce offspring represents an evolutionary paradox, the so-called 'two-fold cost of sex' (Maynard Smith 1976).

Two major models proposed hypotheses to explain why obligate asexuality seldom replaces sexual reproduction in natural population despite its advantage. The mutation-accumulation model (Muller 1964; Kondrashov 1988) suggest that sex is adaptive because it purges the genome of recurrent and harmful mutations. This model requires a high mutation rate, which appears unlikely. On the other hand the ecologically based model postulates that sex is adaptive under variable environments (Maynard Smith 1976) where new gene combinations facilitate adaptation (Maynard Smith 1976). As explained above, parasites represent a selective component of the environment that can vary from one generation to the other and thus would favor sexual reproduction (Maynard Smith 1976; Hamilton 1980; Hamilton et al. 1990; Ebert & Hamilton 1996).

To be stable, sexually reproducing individuals should have more than a two fold fitness advantage over asexuals (Hamilton et al. 1990). This hypothesis has strong theoretical and empirical support (Lively 1987; Peters & Lively 1999). In one of his numerous works on the evolution of sex due to host-parasite interactions, Curtis Lively compared success of parthenogenetic and sexual forms coexisting within a snail species (*Potamopyrgus antipodarum*). He showed that sexual forms had a fitness advantage, attributed to the ability of their genetically diverse offspring to better resist a trematode parasite. Sex was also beneficial for the parasite, facilitating tracking of the common host genotype (Lively 1987; Lively 1992). Models of parasite-host interactions that favor sexual reproduction typically assume that parasite adaptation to one host genotype requires lower fitness of the other host genotypes, consistent with the matching-allele model (see box 3 and Howard & Lively 1994; Peters & Lively 1999).

Box 3: Matching allele model

In host/parasite interactions, a co-evolutionary arms race can 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 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 the matching allele model (Howard & Lively 1994). In this model, matching of parasite and host alleles produces a specific outcome. For example, each host allele may cause recognition and resistance to a matching parasite allele (Frank 2002). Alternatively, each parasite allele might be able to attack only a single host resistance allele. In both cases, each parasite genotype functions as either a resistant or susceptible allele, depending on the host genotype, and vice versa. The matching allele model does not require cost of resistance and virulence to explain the maintenance of high levels of polymorphism (Kniskern & Rausher 2001).

III- Speciation: Another consequence of host immune gene/ parasite co-evolution?

III.1- Species concepts

The most widely applied definition of species refers to the biological species concept, introduced by Dobzhansky and elaborated by Mayr. (Dobzhansky 1936; Mayr 1942; Mayr 1963; see more recently Coyne & Orr 2004): "Species are groups of interbreeding natural populations that are reproductively isolated from other such groups". This definition identifies reproductive isolation as the primary factor to discriminate between species and thus provides a focus on differences that prevent successful interbreeding under natural conditions. It does not include geographical isolation since physical barriers may be temporary. Moreover, it insists on the non natural hybridization. The last point allows meeting the molecular concept of species where species share a common pool of genes (Dieckmann et al. 2004; Ridley 2004). Note that many animal groups also contain groups that hybridize but remain distinct species. How much gene flow is "allowed" between biological species is debatable (Moore & Hendry 2005; Rundle & Nosil 2005; Nosil 2008).

III.2- Speciation mechanisms

III.2.1- Natural selection: a fuel for ecological speciation

Behaviors and morphological characters observed in animals are the result of the history of species and populations. Organisms have evolved through a selective process. When organism's characteristics are favorable for survival and reproduction of individuals in a stable given environment, genotypes underlying these phenotypes tend to spread in the population. This phenomenon of increasing beneficial alleles as a consequence of higher survival and reproduction corresponds to the process of natural selection (Darwin 1859). There are three postulates to the process of natural selection: i) there is variation between individuals for a given trait within a population, ii) there is a coherent relationship between this trait and the ability of individuals to survive (e.g. predation avoidance) and reproduce (e.g. fecundity), and iii) this trait is heritable independently of the fact that the successive generations inhabit the same environment.

Based on the biological species concept, identifying the causes of speciation requires identifying barriers to gene flow between diverging populations and thus for identifying pressures driving natural selection when present. Barriers to gene flow fall into two broad groups: pre-zygotic barriers that act to limit the formation of zygotes, and post-zygotic barriers that act to reduce the fitness of hybrids (Mayr 1942).

III.2.2- Pre-zygotic isolation

Pre-zygotic isolating mechanisms prevent the union of gametes that produce a zygote, and include several types of barriers: 1) mates do not meet. This could be due to seasonal variation or habitat isolation. 2) mates meet but do not mate due to variable reproductive behaviors. Particularly, assortative mating is defined as the occurrence of mating within forms more often than between forms. Such a mating strategy can be the outcome of multiple different premating barriers and, therefore, is a useful way of quantifying pre-mating reproductive isolation. Assortative mating could be a reproductive strategy allowing maintenance of locally adapted pools of genes for instance. 3) mates meet but no sperm transfer occurs. This last mechanism represents a case of mechanical isolation. 4) gametic isolation represent a case where mating occurs but some incompatibility prevents sperm from fertilizing the egg.

III.2.3- Post-zygotic isolation

Post zygotic barriers include reduced hybrid fitness or fertility (Coyne & Orr 2004). These mechanisms can be classified as follows: 1) the egg is fertilized but selective abortion occur. 2) Hybrid offspring experiences reduced viability due to a lack of adaptation to local or current

environmental conditions. 3) Hybrids are viable but are partially or completely sterile and F2 are in some way deficient. Note, the predominant genetic model of the evolution of hybrid incompatibilities proposes that they are a consequence of negative epistasis between alleles at interacting genes that evolved on different genetic backgrounds (Dobzhansky 1936). Identifying these incompatible genes is challenging, thus only five incompatibility loci have been identified (Wittbrodt et al. 1989; Ting et al. 1998; Barbash et al. 2003; Presgraves et al. 2003; Brideau et al. 2006).

III.2.4- Geographic mode of speciation

Very often speciation has been described with regard to geographical differences (e.g. Mayr 1942; Maynard Smith 1966; Dieckmann & Doebeli 1999; Dieckmann et al. 2004; e.g. Bolnick & Fitzpatrick 2007) Fig. 1). Speciation is called allopatric when it occurs as a consequence of physical isolation, i.e. gene flow between incipient species is zero from the beginning (Mayr 1963). If two populations inhabit adjacent habitats allowing gene flow, the speciation is called parapatric (Gavrilets 2003). Another definition of parapatric speciation specifies that gene flow occurs across a spatially restricted contact zone such that only a fraction of each population has a high probability of interacting with immigrants (Smith 1965). Eventually, when reproductive isolation occurs within a single local population, the speciation is called sympatric (Mayr 1942). Such case of speciation is the most debated and splits the scientific community for decades (see for an example Mayr 1963; Maynard Smith 1966; see for an example Barluenga et al. 2006; Schliewen et al. 2006; Fitzpatrick et al. 2008).

Although geographical context is important to understand divergence, to my opinion this context can be better explored by modeling, identifying and measuring gene flow and selective pressure rather than assigning cases to categories like sympatric and allopatric speciation. We will see later in this thesis that potential mechanism driving speciation could be independent of the geographical context.

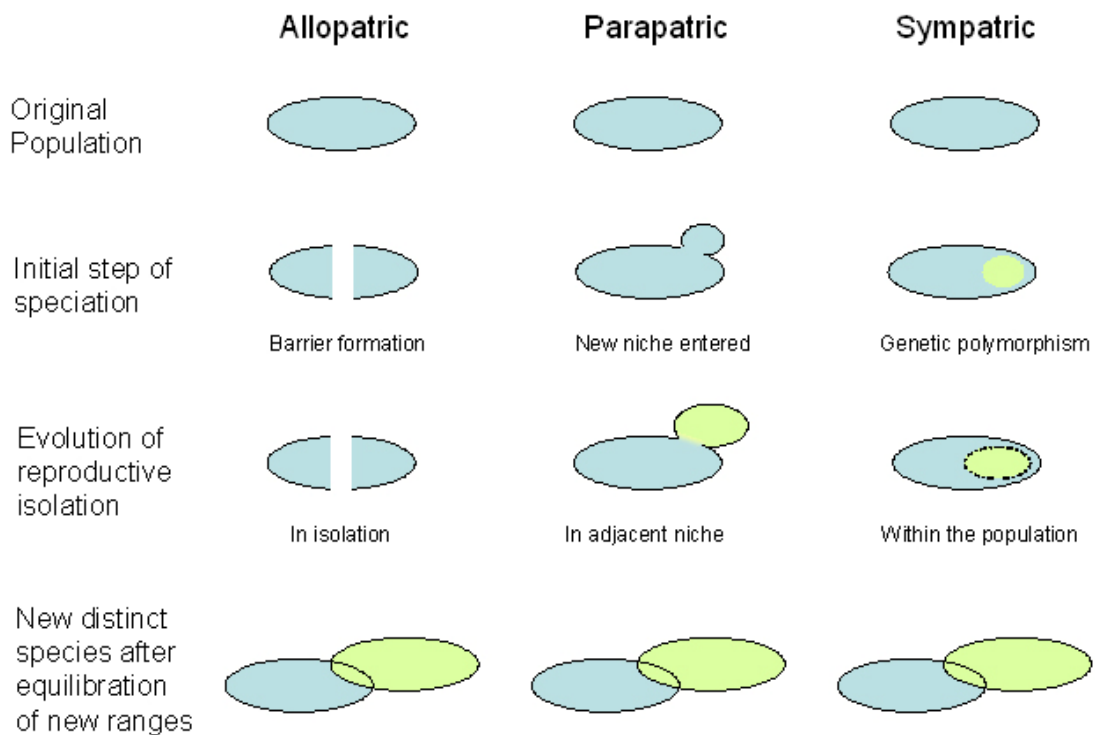


Fig. 1: Schematic description of the different mode of speciation (adapted from biology lecture Dr. Krempels. (see for recent review Butlin et al. 2008; but as well Fitzpatrick et al. 2008))

III.3- The ecological concept of speciation

III.3.1- Definition of ecological speciation

The ecological processes that drive speciation have recently been the focus of intense research (e.g. Hatfield & Schluter 1999; McKinnon & Rundle 2002; Richmond & Reeder 2002; Dieckmann et al. 2004; Dall et al. 2005; e.g. Rundle & Nosil 2005; Ryan et al. 2007). These ecological processes are the interactions between individual organisms and their environment that causes natural selection. Ecological speciation refers to the mechanisms by which reproductive isolation evolves as a by-product of divergent selection on populations resulting from differences in their environment. Ecological speciation is typically accompanied by substantial shifts in phenotype [in this case “phenotype” entails morphological, physiological and resource use traits (Schluter & Rambaut 1996)].

III.3.2- Example - Darwin’s finches

One of the most famous cases of speciation driven by interactions between individuals and their environment are Darwin’s finches (Boag & Grant 1981). Fourteen species of Darwin’s finches live in the Galapagos archipelago (Fig 2). All derived from one common ancestor, which colonized the archipelago ca. 3 million years ago.

Populations of a species on different islands differ in beak size and shape. Variation of this trait exists at both inter- and intra-specific levels. Different beaks have properties of tools of various sizes and shapes that perform different tasks of grasping and crushing. Where beak differences are pronounced, a correspondence between the average size of beaks and characteristics of the diet such as seed size was observed (Abbott et al. 1977; Grant & Abbott 1980; Grant & Grant 1982).

The first hints of the connection between beak shape and food regime were observed by scientists during a severe drought on the island Daphne. During that hot season, 85% of the medium ground finches (*G. fortis*) died. First, small and abundant soft seeds were eaten. The drought prevented the regeneration and re-growth of most of the seed-producing plants. Finches turned towards the large and hard seeds. However, cracking such seeds for the small finches appeared difficult and time consuming if at all achievable. As a consequence, these small birds died at a higher rate than large birds. As a result of this differential mortality, average beak size as well as average body size increased until the rain resumed. 74% of the beak variance and 91% of the body size were heritable, showing that these populations experienced strong natural selection (Grant 1986).

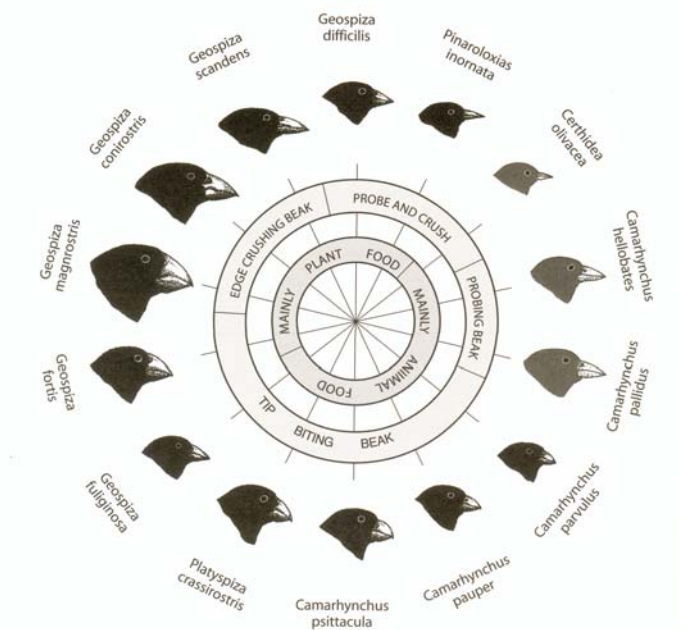


Fig 2: Adaptive radiation

Dispersion of colonizing population, about 1 to 5 My ago among island presenting contrasting habitats and resources favoured speciation and led to 14 morphologically and ecologically different Darwin's finches species. (Picture reproduced with the kind authorization of P. Grant).

III.3.3- When parasites are invoked

Competitive interactions and niche utilization are not the only selective pressures that can cause population divergence. Haldane (1992) and later Hutchinson (1959) pointed out that host-parasite conflicts could generate diversity both within and between species.

Extreme specialization is most common in parasitic species - often populations of parasite are geographically differentiated, with specialization on different hosts in different geographic regions (Thompson 1994). Price (1980) argued that a parasitic lifestyle is likely to lead to population subdivision, geographic differentiation and the formation of sibling species. In turn, host-parasite co-evolution should promote host speciation as well (Haldane 1992). The "speciability" (ability to speciate) in hosts and parasites is influenced by the nature of parasite virulence and host resistance. Under local adaptation, local parasites are more effective than foreign parasites at exploiting hosts, whilst under local mal-adaptation the opposite prevails (Kniskern & Rausher 2001). Under local mal-adaptation, where the host populations are resistant to the local pathogen, migrants or hybrids between local and foreign hosts should suffer from higher parasite pressure. This should promote reproductive isolation among host populations, but not among parasite populations. The opposite would be true under a scenario of local adaptation (Summers et al. 2003). Since host populations generally have to face various infectious agents, these two examples represent two ends of a continuum, where an optimal strategy in resistance for the host and in virulence for the parasite exists.

We have seen that parasitic exploitation has the potential to promote reproductive isolation (Haldane 1992; Turelli et al. 2001). An obvious mechanism is one in which hybrids experience low fitness relative to genotypes that have co-evolved with the specific parasite community (Haldane 1992). Several studies have reported lower parasite resistance in hybrids relative to the sympatric host of the studied parasites (Sage et al. 1986; Moullia et al. 1991; Kalbe & Kurtz 2006). However, in numerous examples of host-parasite interactions, local adaptation of the parasite could not be detected. In several cases even the reverse pattern was found (Ebert & Hamilton 1996; Kaltz & Shykoff 1998). Lajeunesse and Forbes (2002) revealed a regularity in these contradictory findings: parasites with a narrow host range, i.e. those which develop only in one or a few host species tend to become locally adapted, whereas local adaptation is rather unlikely in parasites with a broad host range. Generalist parasites with the potential to infect several different species of host may hardly afford specialization on the locally most common genotype of only one of the many host species (Lively & Dybdahl 2000).

Once more, such results demonstrate that an optimal strategy in host resistance requires trade-offs between resistance to specialist and generalists parasites.

IV- The three-spined stickleback as model organism

IV.1- The three-spined stickleback

The three-spined stickleback (*Gasterosteus aculeatus*) is an ideal model system for studying host-parasite interactions; it is abundant, widely distributed across the Northern hemisphere and easy to collect and rear in lab. But what makes it an exceptional model organism is that its behavior and ecology have been intensely investigated. A Nobel Prize was awarded to Tinbergen's (1973) for his work on stickleback on the elicitation of individual and social behavior patterns. In addition, since 2006, its genome is freely accessible (<http://www.ensembl.org>), facilitating the use of sophisticated molecular techniques.

IV.2.1-Ecology of the three-spined stickleback

In northern Germany, the source of my study populations, as in other populations from our latitudes, the stickleback breeding season starts in early spring, when males display an intense red coloration on their throat (Wootton 1976). At this time, males build a nest out of algae and plant material. To assure resistance and maintenance of their nest, males secrete glue made of glycoprotein onto the nest. As soon as a female has produced a mature egg clutch, she approaches the nest of a male, and a complex mating behavior between both partners starts (Wootton 1976). After courtship, the male leads the female to the nest. Once the female has spawned, the male chases her away and provides paternal cares until the eggs hatch. Paternal care consists of ensuring oxygen supply by fanning and by removing molded eggs. However, competitor males might disturb such sequence. Indeed, sneaking behavior has been reported (Wootton 1976). It is expected that low quality males, which are not able to establish a territory, will attempt to steal fertilizations. The stickleback populations we investigated have only one reproductive period after their first winter. After reproduction, the generation overlap is very short and later in the year it becomes difficult to find fish from the parental generation. This offers a fantastic system to study mate choice and its consequences on lifetime reproductive success.

Reproductive behaviors in both the male (competition for territory, nest building, secondary sexual trait display, and paternal care) and the female (repeated production of egg clutches, finding mates) are costly fitness components that might be affected by parasitization and thus be related to MHC genes.

Additionally, the sticklebacks have undergone drastic adaptive radiation since the last glacial period (Bell & Foster 1994). In this relatively short time period of 10.000-15.000 years, isolated populations have experienced dramatic changes, with different populations showing pronounced changes in body size, pattern and number of lateral plates as well as behavioral differences (e.g. Bell & Foster 1994; Schluter & Rambaut 1996; Schluter 2000; Boughman

2001; Schluter 2001; Colosimo et al. 2005; Boughman 2007). All these changes have been proposed to result from habitat adaptation. However, very little is known about the role of parasites in host adaptation and how host-parasite arms races might have shaped immune genes and the distribution of populations.

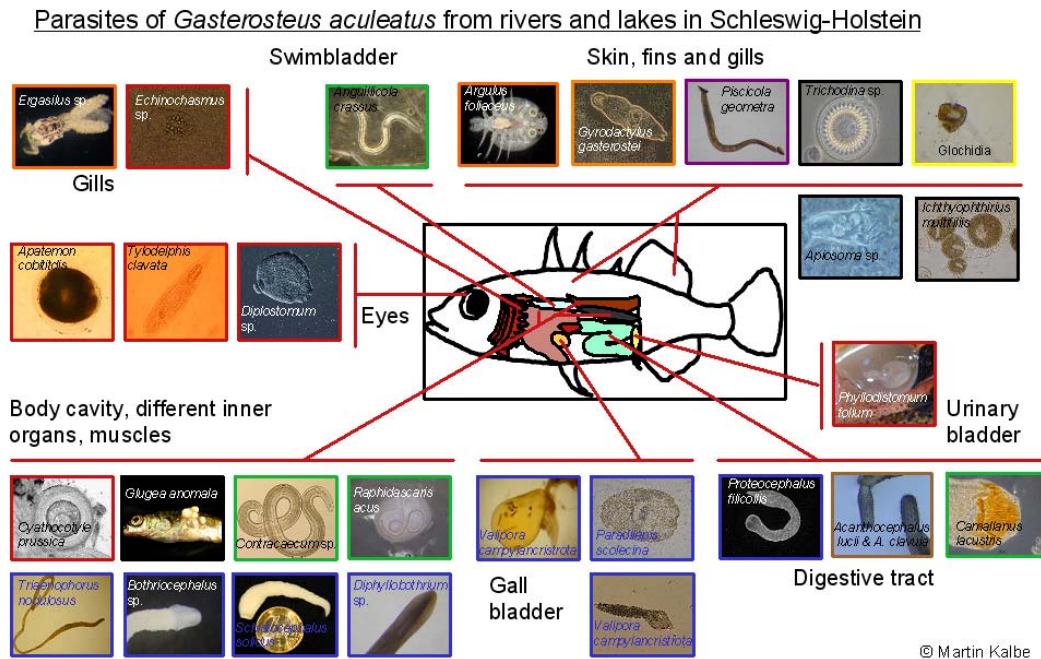


Fig. 3: Three-spined sticklebacks host an amazing diversity of parasite species. Besides viruses, bacteria, fungi and protozoans, macroparasites are probably very important agents driving the evolution of sticklebacks

Specifically, genetic studies revealed that habitat differences contributed most to the genetic variation among three-spined stickleback populations from northern Germany (Reusch et al. 2001b). Parasite communities differ among fish habitats in the same drainage system (lake versus river) (Kalbe et al. 2002) Fig. 3). Eventually, fish were screened for MHC diversity. Wegner *et al.* (2003b) showed that where fish were exposed to a more intense parasite pressure, the MHC diversity was higher. So far, however, the relationship between the pool of MHC alleles and habitat differences between habitats has not been characterized.

IV.2.2- MHC in the three-spined stickleback

MHC in the three-spined stickleback has been investigated in a number of studies. The first studies were performed on mate choice. Female sticklebacks were experimentally shown in a dichotomic mate choice to favour males with which they share an intermediate MHC diversity (Reusch et al. 2001a; Aeschlimann et al. 2003). Later, Milinski *et al.* (2005) revealed that females are able to assess MHC make-up through peptides present in odor signals. Such

mate choice is consistent with a lower parasite burden for fish harboring an intermediate MHC diversity (Wegner et al. 2003a; Wegner et al. 2003b) but as well a better activation of the innate immune system (Kurtz et al. 2004).

In the past, the number of MHC class IIB loci in the stickleback had been reported to be as high as 6 (Sato et al. 1998), but this was recently corrected to 2-4 (Reusch & Langefors 2005). Potentially due to recent origin of locus duplication or ongoing inter-locus recombination (Reusch et al. 2004), it is not possible to address these loci singly and therefore several alleles per individual have to be differentiated, independent of the genotyping technique used

IV.2.3-MHC typing

Genotyping methods for such variable and often duplicated loci as MHC genes are scarce and difficult to optimise. Unfortunately, except in mammalian species, it is often impossible to target single locus because of low conservation in MHC structure as seen in a lot of species (Edwards et al. 1995). This is particularly true in sticklebacks, due to recent duplication and/or on-going inter-locus recombination (Reusch et al. 2004), it is not possible to address these loci singly and therefore several alleles per individual have to be differentiated, independent of the genotyping technique used. For the studies reported in this thesis, we have used two different indirect typing methods. Such methods are cost effective and allow high throughput screening. The SSCP (Single Strand Conformation Polymorphism) method has been efficiently used in the three-spined stickleback in numerous studies (Reusch et al. 2001a; Aeschlimann et al. 2003; Wegner et al. 2003a; Wegner et al. 2003b; Milinski et al. 2005; Wegner et al. 2008). However, this method reached its limits with tracking specific alleles between cohorts or populations. Lenz *et al.* (in prep- See Appendix I) have solved this problem by adapting a published method (Baquero et al. 2006) to our model organism- RSCA (Reference-mediated Conformation Analysis). In combination with a comprehensive plasmid library of MHC class II B alleles, it is now possible to not only identify a specific allele but also to obtain its nucleotide sequence. The optimization of this technique provides a diagnostic tool to compare allelic pools between populations and cohorts.

In this thesis, I investigated how host immune system/ parasite co-evolution affected fitness related traits, mating decisions and reproductive success. Based on this knowledge, I investigated whether host/parasite co-evolution in different environments could ultimately result in population divergence and speciation.

Thesis Outline

In this thesis, I (with colleagues) brought together the role of natural and sexual selection on the evolution of the genes of the Major histocompatibility Complex (MHC). To this end, we investigated the fitness consequences of parasite infection on both males' and females' fitness traits in a laboratory-study (Chapter I). Then, in two independent field-study experiments, we investigated MHC based female mate choice (Chapters II and III). In the following chapter (IV) we used the knowledge acquired on MHC-related fitness traits to predict a potential speciation mechanism mediated by these immune genes and their response to being parasitized. The fifth chapter presents correlative evidence for the proposed speciation mechanism, while the next chapter (VI) reports an empirical test of it. Finally, the last chapter reports an experimental trial to test negative-frequency-dependant selection by parasites. Except for the fourth, all chapters are structured in abstract, introduction, methods, results and discussion. In cases where the chapter has already been published, the section sequence of the original article has been maintained (Chapters 1 & 4). This outline gives an overview of the individual studies. All studies presented here are a result of collaboration with several colleagues (Table 1).

Chapter I: Immuno-ecology of the three-spined stickleback

Since parasites impose costs to their hosts, they potentially reduce fitness. MHC genes play a key role in adaptive immunity of vertebrates and are thus tightly linked to parasite resistance. With wild-caught three-spined sticklebacks (*Gasterosteus aculeatus*) we investigated the relation between individual reproductive fitness traits, parasitization and both MHC class I and II diversities. As male fitness trait, we focused on the quality of the nest and the intensity of the red breeding coloration. Producing and maintaining a high-quality nest requires energy and is probably dependent on the male condition, as is breeding coloration. As a female trait, we followed the number of egg laying events; a crucial parameter of the reproductive success

Chapter II: MHC-based reproductive success in three-spined stickleback

Previous studies on three-spined sticklebacks have reported multiple advantages to fish with intermediate MHC diversity: lower parasite burden, higher body condition, best nest quality and greater immune activation. The reality of active female mate choice is now unquestioned and should lead better-adapted individuals to maximize their Darwinian fitness. To test this hypothesis, we measured the lifetime reproductive success of sticklebacks under semi-natural conditions by genotyping eggs. We analyzed mating combinations and individual reproductive output of laboratory-bred sticklebacks. Within each of six enclosures, we used siblings (8 males and 8 females) to reduce the influence of non-MHC genes (genetic background) and

fish therefore differed almost completely with respect to their MHC genotype. We predicted that fish with an intermediate MHC diversity should exhibit the highest reproductive success.

Chapter III: Good genes transmission, maintenance of polymorphism and speciation are consequences of a MHC-based assortative mating.

In the previous study we have shown that laboratory-bred female three-spined sticklebacks exert an MHC-based mate choice under semi-natural condition. However, little is known about mate selection in wild-caught populations. Thus, during the reproductive period, wild-caught fish were naturally and continuously confronted with their natural sympatric parasites in the same enclosure system as previously described. Here, we aimed at extending the conclusions derived from laboratory-bred fish. In this study, using a newly developed MHC typing method, we also questioned the role of single MHC haplotypes in the female mating decision. We stocked the enclosure with two different male densities in order to be as close as possible to natural conditions, where females have access to a high number of potential mates.

Chapter IV: An ecological mechanism for speciation orchestrated through MHC/parasites interactions.

In the previous study, we showed that females mated assortatively with regard to MHC genes, potentially to not disrupt locally-adapted genetic combinations. Based on this result, in the present work, we summarized theoretical discussions and developed a mechanism for speciation that is both initiated and strengthened by parasite communities acting on immune genes. In contrasting niches, parasite communities differ and also should the pools of adapted MHC alleles. We predict mate choice to be directed towards the best-adapted MHC genotype. This should favor local adaptations and should accelerate separation of both populations: thus immune genes would act as ecologically-relevant pleiotropic speciation genes. The crucial underlying idea is based on the small optimal individual number of MHC loci: each individual display a reduced subset of the many MHC alleles present in the population, adapting it only to a local pathogenic environment.

Chapter V: Tracking the first steps of speciation in different three-spined stickleback ecotypes

As proposed in the previous chapter, MHC local adaptation may promote the evolution of assortative mating in host populations as a mechanism through which genetically locally-adapted offspring can be produced. To test for evidence of this mechanism, we sampled three-spined stickleback populations from a lake and a connected river. Such ecosystems have already been shown to support distinct parasite communities. We assumed that contrasting parasite communities should drive evolution of contrasting MHC variant pools between

ecotypes. Using the same population, we also performed olfactory based mate choice, to test for assortative mating within ecotype based on a genetic basis of the olfactory cues, reflecting potentially MHC genotypes.

Chapter VI: Testing MHC adaptation: a field experiment

In the previous chapters (IV & V), we suggested that MHC genes could act as a “magic trait” adapting individuals to their local environment and influencing mate choice. However, the presence of local MHC adaptation has so far not been experimentally tested. For the present study, we crossed three-spined sticklebacks from the same river and the same lake habitat as previously used. The fish from these populations display almost totally distinct MHC allele pools. With a precise breeding design, we obtained a second fish randomized for the genetic background but segregating into pure river, pure lake and two hybrid MHC genotypes. Using replicated cages, we used a double common garden experiment where fish were exposed simultaneously to lake and to river pathogenic conditions during 10 months.

Chapter VII: Testing experimentally negative frequency dependent selection by parasite in the three-spined stickleback

Both parasites and mate choice selection are thought to maintain polymorphism at the pleiotropic MHC immuno-genes. Although the role of negative-frequency dependent selection by parasites has often been invoked in this, to date, no empirical support has been provided for it. Individuals carrying specific MHC allele conferring resistance against currently predominating parasites should perform better and should be favored during female mating decisions, and resistant alleles should therefore increase in frequency. Dynamic changes in frequency are linked to the reduced individual optimal number of MHC loci, which cannot display the extreme MHC allelic diversity present in the population. Here, we detail an experiment to test for negative frequency-dependent selection, by not only including parasite pressure but also competition among males and mate choice. We created replicated populations each formed by 6 different laboratory-bred families in equal proportions. Three experimental populations were exposed to *Camallanus lacustris*, while the three others will be confronted with *Anguillicoloides crassus*. Letting the system evolve in outside controlled tanks, we assumed that females would mate with partners possessing a resistance allele against the experimental parasites leading this allele to increase in frequency in the following generation. After raising the collected egg in the lab, we confronted the new fish generation with both parasites. We hypothesized that fish from parents that encountered one parasite, e.g. *Camallanus lacustris*, will be less heavily infected by this parasite than those coming from parents that encountered *Anguillicoloides crassus* and vice versa.

Table 1: Contributions to individual Chapters

	Chapter I	Chapter II	Chapter III	Chapter IV	Chapter V	Chapter VI	Chapter VII
Idea & experimental design	MM, SWG	MK, MM, TBHR	MK, MM	CE , MM	CE , MM	CE , MM, TLL	CE ,MM, TLL
Research performance	HS, KK, MM, MK, SG, TBHR,	CE , ID, MK, MKW, RDS	CE , MK, SY, TLL	AT, CE , MM, TLL	CE , CH, MK, TLL	CE , CH, TLL, MK	CE , MK, JS, TLL
Data analysis	CE , IJ, KK	CE	CE	-	CE	CE	CE
Writing Manuscripts	CE , IJ,MM	CE , MK	CE	CE	CE	CE , TLL	CE ,TLL

AT: Arne Traulsen; **CE: Christophe Eizaguirre**; CH: Chris Harrod; HS: Helmut Schaschle; ID: Ilka Dankert; JS: Joern Scharsack; KK: Kerstin Krobbach; MK: Martin Kalbe; MKW: Matthias K. Wegner; MM: Manfred Milinski; RDS: Ralf D. Sommerfeld; SWG: Sian W. Griffith; SY: Sarah Yeates; TBHR: Thorsten B. H. Reusch; TLL: Tobias L. Lenz; -: not applicable.

Chapter I

Individual MHC class I and MHC class II *B* diversities are associated with male and female reproductive traits in the three-spined stickleback

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Abstract

Genes of the major histocompatibility complex (MHC) are indispensable for pathogen defence in vertebrates. With wild-caught three-spined sticklebacks (*Gasterosteus aculeatus*) we conducted the first study to relate individual reproductive parameters to both MHC class I and II diversities. An optimal MHC class IIB diversity was found for male nest quality. However, male breeding coloration was most intense at a maximal MHC class I diversity. One MHC class I allele was associated with male redness. Similarly, one MHC class IIB allele was associated with continuous rather than early female reproduction, possibly extending the reproductive period. Both alleles occurred more frequently with increasing individual allele diversity. We suggest that if an allele is currently not part of the optimum, it had not been propagated by choosy females. The parasite against which this allele provides resistance is therefore unlikely to have been predominant the previous year – a step to negative frequency-dependent selection.

Introduction

The major histocompatibility complex (MHC) is a gene dense region of all jawed vertebrates that plays a key role in answering important questions in fields of evolutionary ecology such as parasite-mediated selection (e.g. (Paterson et al. 1998; Langefors et al. 2001; Grimholt et al. 2003; Wegner et al. 2003a; Wegner et al. 2003b; Bonneaud et al. 2005; Harf & Sommer 2005; Madsen & Ujvari 2006); reviews in (Hedrick 1999; Milinski 2006) and sexual selection (e.g. (Yamazaki et al. 1976; Potts et al. 1991; Reusch et al. 2001a; Aeschlimann et al. 2003; Olsson et al. 2003; Milinski et al. 2005; Bonneaud et al. 2006a), reviewed in (Penn & Potts 1999; Milinski 2006). Classical MHC genes are central to the adaptive (or acquired) immune system of vertebrates and contain the most polymorphic gene loci known in vertebrates (e.g. Janeway et al. 1999). MHC molecules present pathogenderived peptides to T cells that subsequently initiate a specific immune response. There are two classes of MHC molecules, class I and II, that are found on different linkage groups in bony fishes, in contrast to higher vertebrates, thus recombine freely. Whereas MHC class I molecules bind peptides derived from intracellular pathogens, e.g. viruses, MHC class II molecules bind peptides from extracellular pathogens, e.g. macroparasites. To be able to fight a broad range of pathogens, one would expect selection to have favoured high individual MHC diversity, i.e. high individual MHC heterozygosity. However, an increased number of MHC variants may not only bind more foreign peptides but also a greater variety of self-derived peptides raising the risk of autoimmune responses. T-cell clones that recognize self-peptides bound to MHC molecules (class I and class II) are thus eliminated from the initially highly variable repertoire, thereby reducing the T-cell clone repertoire that is ultimately available for parasite recognition (Lawlor et al. 1990; Goldrath & Bevan 1999). A balance between the two opposing selective forces of either broader pathogen detection or increased T-cell clone elimination may lead to an optimal rather than a maximal individual MHC diversity (Nowak et al. 1992; De Boer & Perelson 1993; Borghans et al. 2003), review in (Milinski 2006).

Teleost fish are ideal for the study of MHC class I and II diversities, as, unlike other vertebrates, the MHC class I and class II regions are not linked, therefore allowing the effects of each class to be tested independently (Sato et al. 1998; Stet et al. 2002). Three-spined sticklebacks (*Gasterosteus aculeatus*, Linnaeus, 1758) have probably up to 12 MHC class I and six MHC class IIB loci (Sato et al. 1998), that are extremely similar to one another due to interlocus gene conversion and/or recent duplication (Reusch et al. 2004; Reusch & Langefors 2005; Schaschl & Wegner 2007). The different number of allelic variants can be achieved through heterozygosity, or possibly, through different haplotype copy number of MHC genes. In the stickleback, evidence for the existence of an optimal (about six alleles) rather than a

maximal (12 alleles) individual MHC class IIB diversity is mounting: the mean individual diversity in a natural population was found to be 5.8 alleles (Reusch et al. 2001a) and wild-caught sticklebacks carried a minimal parasite burden when they had 5.2 MHC class IIB alleles (Wegner et al. 2003b; Waga et al. 2007). Moreover, individuals with an intermediate number of MHC class IIB alleles were infected least after experimental exposure to a defined parasite dosage (Wegner et al. 2003a; Kurtz et al. 2004). In this study, we investigate both MHC class I and II genes. Previous MHC-related studies in the stickleback have been restricted to MHC class IIB.

As in other vertebrate taxa (reviews in (Penn & Potts 1999; Penn 2002; Milinski 2006), mate choice in the three-spined stickleback is related to MHC (Reusch et al. 2001a; Aeschlimann et al. 2003; Milinski 2003; Milinski et al. 2005). In contrast to several studies, which found a preference for MHC dissimilar mates (e.g. Yamazaki et al. 1976; Wedekind et al. 1995; Olsson et al. 2003; Richardson et al. 2005), stickleback females did not prefer MHC-dissimilar mating partners to MHC-similar ones (Reusch et al. 2001a). Rather, females chose partners such that, in self-reference to the number of their own alleles, the mean MHC IIB allele diversity of the offspring would be close to the optimal number (Aeschlimann et al. 2003; Milinski 2003). Female sticklebacks base their MHC-related mating preference on odor signals, with MHC ligand peptides being the polymorphic odor cue (Milinski et al. 2005). However, stickleback mate choice depends also on male secondary sexual traits like breeding coloration (McLennan & McPhail 1990; Milinski & Bakker 1990a; Bakker & Milinski 1991; Milinski & Bakker 1992), nest quality (Barber et al. 2001b) and display behaviour, i.e. the zigzag dance (Rowland 1995). Females are expected to assess their mates by traits honestly reflecting their condition (Zahavi 1975; Hamilton & Zuk 1982). Indeed, the intensity of the male red breeding coloration has been shown to reveal condition, parasitization and parasite resistance (Milinski & Bakker 1990a). As expected, females prefer redder, hence unparasitized, males (Milinski & Bakker 1990a). Despite the connection between the immune status and breeding coloration of males, male redness has not yet been related to MHC-based pathogen resistance as suggested by Aeschlimann *et al.* (2003) and Milinski *et al.* (Milinski 2003; Milinski 2006). Similarly, other fitness-related traits such as male nest quality or the number of egg clutches a female is able to spawn might reflect an individual's MHC-dependent immune status. We would expect to find these traits maximized in individuals with an optimal MHC class IIB diversity and/ or specific MHC alleles that provide resistance against the currently predominant pathogens. It is not yet known whether MHC class I diversity is correlated with fitness-related traits in the three-spined stickleback. However, following the hypothesis of two opposing selective forces, we would also expect an optimal number of MHC class I alleles to be related to high performance in the traits under investigation. Supporting evidence comes from two studies on house sparrows and

water pythons, which found an intermediate MHC class I individual diversity to be optimal with respect to fitness relevant traits (Bonneaud et al. 2004; Madsen & Ujvari 2006).

In the present study, we related individual diversity of both MHC class IIB and MHC class I to relevant reproductive traits of the three-spined stickleback, namely male nest quality, the intensity of male breeding coloration and the number of egg clutches spawned by females. MHC-based pathogen resistance might be particularly important under poor food conditions as tradeoffs are often weaker or sometimes not even visible at all under good environmental conditions (Stearns 1992). If food is limited, a potentially costly suboptimal immune response might not be compensated for by a higher food intake. This is the first study to investigate MHC class I with respect to reproductive parameters of the threespined stickleback.

Materials and Methods

Study animals. Three-spined sticklebacks were caught in the Große Plo“ner See (Northern Germany) in December 2003. In the laboratory, they were kept first under spring temperature and light conditions and then under summer conditions (18 °C, 16 : 8 h light : dark cycle) until they achieved reproductive condition. Each fish's length (to the nearest mm) and weight (to the nearest mg) were measured 6–7 weeks after being caught (when the fish were transferred from spring to summer conditions). A body condition factor was calculated as $100W/L^b$ where W is the fish weight in g, L the fish length in cm and b the regression coefficient calculated from the logarithm transformed values of length and weight (Frischknecht 1993). In the following, this body condition index is referred to as initial body condition. Under summer conditions, fish were fed ad libitum with frozen chironomid larvae and additional live food every day (well-fed group) or only frozen chironomid larvae once every second day (poorly fed group) without knowledge of the fishes' MHC identity. Individual fish were randomly assigned to the two food groups.

MHC class I and class II genotyping. Genomic DNA was extracted from part of a single dorsal spine from each individual fish using a DNA extraction kit (Invitex, Berlin, Germany). The MHC class IIB exon 2 (b1 domain) diversity was determined by using capillary electrophoresis (CE) single-strand conformation polymorphism (SSCP) as described in Binz *et al.* (2001), with modifications as described in Reusch *et al.* (2001a). MHC class II loci are extremely similar to one another (< 2% nucleotide divergence, Reusch et al. 2004) due to interlocus gene conversion and/or recent duplication (Reusch & Langefors 2005). Recently, CE-SSCP genotyping has been extended to determine individual MHC class I exon 2 (a1 domain) diversity in the three-spined stickleback (Schaschl & Wegner 2006). Like for MHC

class II, MHC class I genes are generated probably by repeated and recent gene duplication (Schaschl & Wegner 2007). Thus, it was not possible for either of the two classes to devise a PCR strategy that is able to amplify a single locus only.

Nest quality, male breeding coloration and number of egg clutches. Nests were classified according to five categories: 0 = no nest; 0.5 = nest messy, no visible entrance; 1 = nest messy, entrance vaguely visible; 1.5 = nest neatly glued, but entrance only vaguely visible; 2 = nest neatly glued and entrance clearly visible. The rationale behind this classification was to assess whether and how well the nest was maintained, i.e. if the filaments of the nest were glued regularly and if the entrance was maintained by the male performing the 'creeping-through' behaviour (Wootton 1976). Male sticklebacks use a secretion produced in their kidneys to glue their nest and thus keep it in shape (Wootton 1976). The only vaguely visible entrance represents an opening that is not regularly used and glued. All nests were assessed by the same person without knowledge about the males' MHC genotype.

The red breeding coloration of males was analysed 1 week after the assessing period. Males were photographed ventrally (without knowledge of the males' MHC genotype) after having been stimulated with a ripe female for 15 min the previous day. The males were held in place using a sponge in a water-filled plastic box which had a window of filter glass (high-resolution skylight filter; Hama, Monheim, Germany). The pictures were taken within a dark box using a digital camera (Olympus E20-p; Olympus Corporation, Tokyo, Japan) with a 36-mm macrolens. Shutter speed was 1/60 s with aperture 7. For illumination, we used four cold lights (KL 1500 LCD; Leica; Solms, Germany) with 3300 K colour temperature. An intensity analysis of the red coloration was performed with IP Lab 3.6.2 for Mac OS 9.2.2 (Scanalytics, Inc., Rockville, MD, USA). On each picture, we selected a defined area of the throat covering the majority of the red parts. For this area, a mean red intensity was determined using the R/RGB model [8-bit red–green–blue colour model, i.e. values 0 (lowest intensity)–255 (highest intensity) for each of the colour channels], i.e. each pixel's red colour intensity was divided by the pixel's total colour intensity to be independent of brightness (Frischknecht 1993). All pictures were measured twice in random order, on two consecutive days by the same person. The marking of the area and the resulting red values were highly repeatable: 95.9% for the marked area as well as 99.98% for the mean coloration.

During a 25-day period, starting 4 weeks after fish had been placed under summer conditions, each female's gravidity was assessed (without knowledge of the females' MHC genotype) every day except days 15, 20 and 24. A female was assumed to have spawned overnight if

she had been assessed as being very ripe on one day but not on the following day. It is known that female sticklebacks spawn their eggs when gravid even in the absence of males.

Parasites. Males were dissected and screened for parasites without knowledge of the males' MHC genotype. As not all dissections could be performed on the same day, only parasite species that could not be eliminated by the host were counted. These included the three digenean trematodes *Diplostomum* sp. (Nordmann, 1832), *Cyathocotyle prussica* (Muhling, 1896) and *Echinochasmus* sp. (Dietz 1909) and the nematode *Contracaecum* sp. (Railliet & Henry, 1915). Individuals of the respective parasite species were counted and an overall parasite load was calculated for each fish using the following approach (Rauch et al. 2006b): for each parasite species, a relative number of individuals per fish was determined (number of individuals per fish divided by the maximum number of individuals of that respective species found on a single fish). The relative numbers of the four parasite species were summed up and averaged over the four species. This index allows parasite burden to be quantified and thus, comparisons to be made between individuals.

Statistical analysis. All statistical analyses were conducted in R statistical package version 2.3.0. Post hoc tests are reported directly in the respective results section. To test for significant correlations between the fishes' (males and females) initial body condition and their individual MHC class I and MHC class IIB diversities, we conducted two independent regressions incorporating a linear as well as a quadratic term in each analysis (function LM).

- **Male traits.**

First, a multiple correlation was performed to remove potential co-linearities in the models. As initial body condition was correlated with food group, this variable is expressed as the residuals from this regression in all following analyses.

o **Nest quality.**

Males were tested for significant effects on nest quality with an ordinal logistic regression incorporating food group, number of MHC class I alleles, number of MHC class IIB alleles, parasite load and initial body condition [function polr, (Ananth & Kleinbaum 1997)(choice of model); (Fox & Andersen 2004)(methods and results report)]. We also included all possible two-way interactions with food group in the model. We then performed independent ordinal logistic regressions to test for an MHC class I and class IIB optimum.

- **Breeding coloration.**

An analysis of covariance (ANCOVA) was performed to test the effect of the above-mentioned factors on male breeding coloration. Independently, the relationship between the number of MHC variants (class I and IIB) and redness was also considered as quadratic function. To test whether the difference in colour intensity between the reddest and the palest quarters of the test fish is linked to a difference in their MHC class I allele composition, we performed an analysis of similarity (ANOSIM, (Clarke & Gorley 2006)). We based the similarity matrix on the Bray–Curtis index (Bray & Curtis 1957), for the choice of the index see (Clarke et al. 2006). Afterwards, a SIMPER test was conducted to estimate whether specific MHC variants contributed to the difference between the two groups. Finally, an ANOVA was performed to test for significant effects of the identified MHC variants on male redness.

- **Female traits**

- **Number of egg clutches.**

An ANCOVA was conducted to analyse the influence of food group, individual allele diversity of MHC class I and class IIB as well as all possible interactions with food group on the number of egg clutches a female spawned. We also tested for quadratic correlations between individual diversity of MHC class I and IIB and the number of egg clutches spawned. To see whether the possession of a specific MHC class IIB allele variant accounted for differences in spawnings at the end of the reproductive period, we conducted an ANOVA. For an analysis of the course of reproductive investment across the 25-day breeding period, repeated measurement MANOVAs were performed with time as the repeated factor and the possession of the identified allele and food group as between-subject variables. To obtain the respective significance levels, repeated measurement MANOVAs were conducted on the respective sub-samples with time as the repeated factor and the possession of the identified allele as between subject variable or just with time as the repeated factor.

Results

The data set consisted of 191 fish (95 males and 96 females). Not all parameters considered were available for all fish resulting in variable sample sizes. For MHC class IIB a mean individual allele diversity of 5.6 ± 0.1 SE was calculated with a median number at six alleles. The mean individual allele diversity for MHC class I was 7.1 ± 0.1 SE with seven being the most frequent individual allele number.

In both, males and females, initial body condition was significantly correlated with a quadratic function of individual MHC class IIB diversity (Regression, $F_{1,173} = 4.131$, $P = 0.044$, $y = 1.31 + 0.11N_{\text{MHC II}} - 0.009N_{\text{MHC II}}^2$) with a maximum calculated for individual diversity of 6.23 alleles.

- **Male traits**

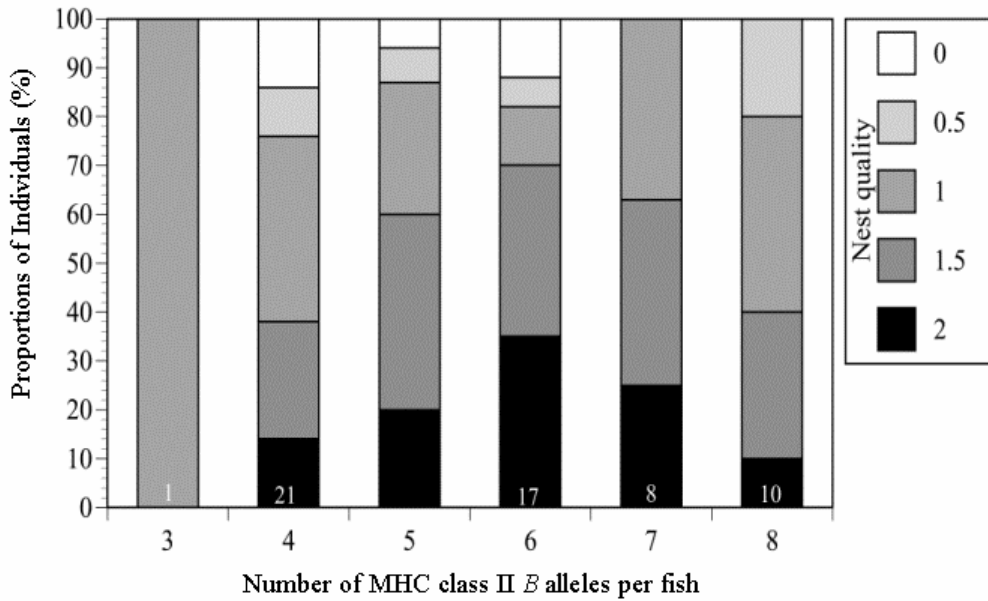
o **Nest quality**

Nest quality was significantly associated with a quadratic function of individual MHC class IIB diversity [Ordinal logistic regression, $N = 72$, value = 0.269, $SE = 0.119$, $X^2_{1,71} = 5.066$, $P = 0.0244$, Table 1(a1)]. Most individuals maintaining a high-quality nest carried an intermediate number of MHC class IIB alleles, whereas most individuals with a low or high allelic diversity had nests of lower quality (Fig. 1). Males possessing six alleles had the highest proportion of nests of the highest quality, depicting an optimum at six alleles (Fig. 1).

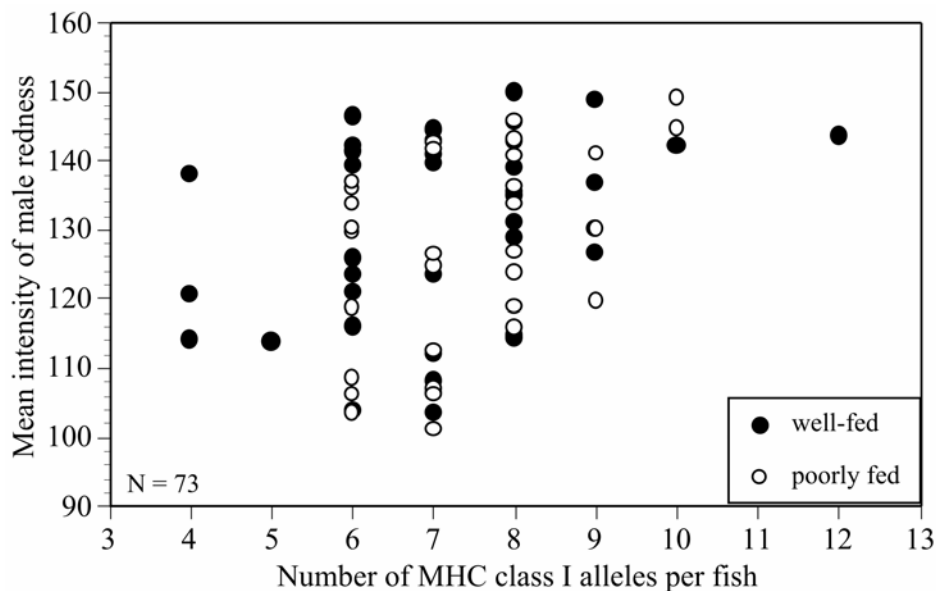
There was a significant interaction effect between food level and parasite load on nest quality [ordinal logistic regression, $N = 72$, value = 0.516, $SE = 0.253$, $X^2_{1,63} = 4.143$, $P = 0.042$, Table 1(a1)]. This resulted in decreasing nest quality with increasing parasite load when fish were under restricted food conditions, whereas parasite load did not have a detectable impact when fish were under good food conditions.

o **Breeding coloration**

Male breeding coloration was not significantly correlated with the quadratic term of either MHC class I or MHC class IIB diversity (regression, $F_{1,70} = 0.749$, $P = 0.390$, and $F_{1,70} = 0.030$, $P = 0.862$). However, males were more intensely coloured with an increasing number of their MHC class I alleles [ANCOVA, $F_{1,68} = 8.528$, $P = 0.005$, Fig. 2, Table 1(b)]. This can be expected if specific alleles that are not part of the optimum allow for high redness. To search for such alleles we compared the reddest quarter of males with the least coloured quarter and found that these two groups differed significantly in their MHC class I allele composition (ANOSIM, $N = 40$, global $R = 0.073$, $P = 0.033$). One specific MHC class I allele contributed most to this difference SIMPER test, 6.85%). Therefore, we tested whether the presence of this allele was related to a more intense coloration performing an ANOVA on redness as a function of this allele. Males were more brightly coloured when they carried the specific allele ($F_{1,44} = 4.5166$, $P = 0.039$). To test whether the likelihood of possessing this allele indeed increased with the number of alleles per individual, we performed a binomial logistic regression on the whole population for each of this allele. The presence of this MHC class I variant increased with an increasing individual MHC class I diversity [GLM (binomial, logit link), $N = 176$, $X^2_{1,191} = 32.963$, $P < 0.0001$].



Chapter I Fig. 1: Proportion of male sticklebacks maintaining nests of different qualities ranging from 0 (no nest) to 2 (high nest quality) in relation to individual MHC class IIB allele diversity. See text for details on how nest quality was assessed. Sample sizes are given at the bottom of the bars.

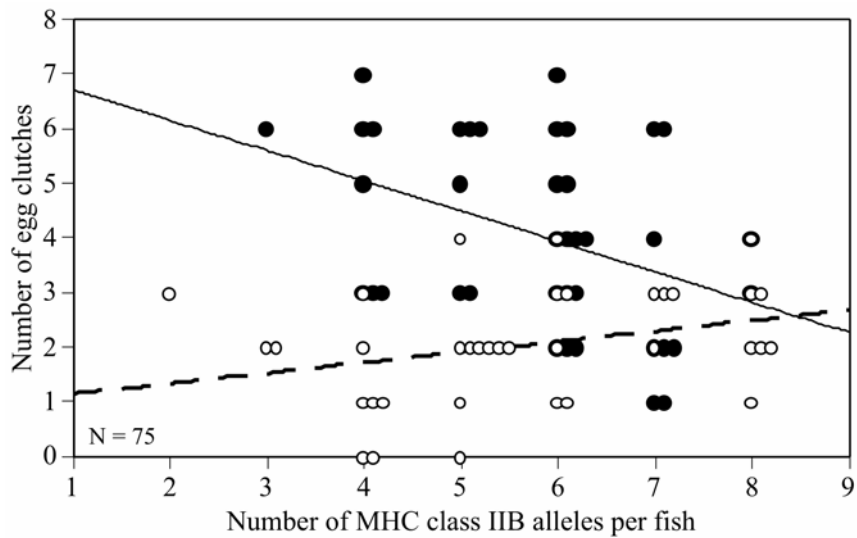


Chapter I Fig. 2: Mean intensity of male breeding coloration in relation to individual MHC class I allele diversity. Intensity values are expressed according to the 8-bit mode, i.e. within a range of 0–255 with 0 representing the lowest and 255 the highest intensity.

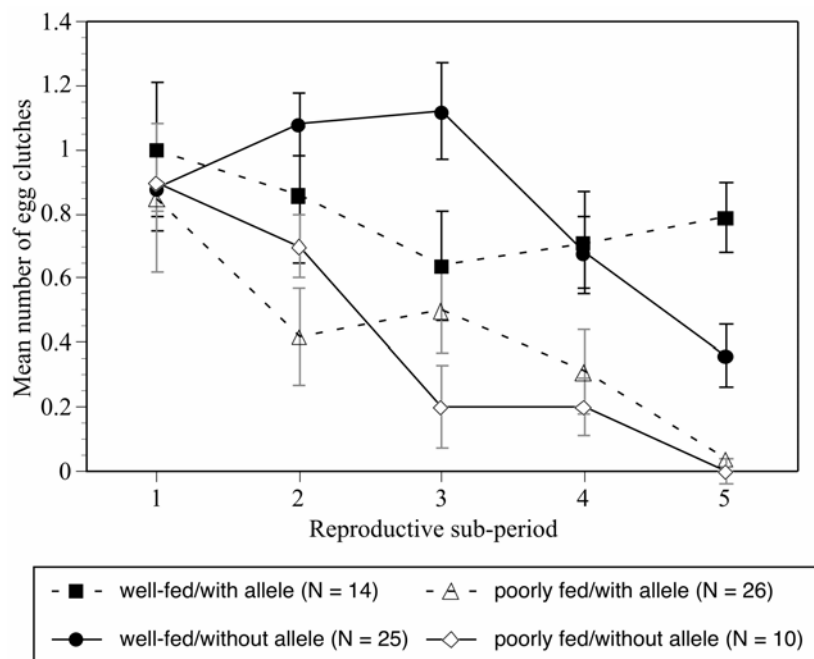
- **Female traits**
 - o **Number of egg clutches**

The quadratic terms of neither individual MHC class I nor MHC class IIB diversity correlated significantly with the number of egg clutches a female spawned (ANCOVA, $F_{1,72} = 0.085$, $P = 0.771$ and $F_{1,72} = 0.319$, $P = 0.574$ respectively). However, females under good food conditions spawned significantly more egg clutches than poorly fed females [ANCOVA, $F_{1,69} = 44.031$, $P < 0.0001$, Figs 3 and 4, Table 1(c)]. It seems that it is not current food availability alone that determines the number of egg clutches a female spawns. We found a significant interaction between food level and individual MHC class IIB diversity on the number of egg clutches (ANCOVA, $F_{1,69} = 9.921$, $P = 0.002$, Fig. 3). Under good food conditions females spawned fewer clutches the more MHC class IIB alleles they carried, whereas the number of clutches spawned by poorly fed females was independent of allelic diversity. We tested whether this result can be explained by a conditional strategy to spawn either early or continuously. We divided the observation time into five subperiods consisting of 5 days each to compare the MHC class IIB composition of those females that spawned in the last subperiod ($N = 20$) with that of females that did not ($N = 19$). The analysis of similarity did not show any significant differences in the MHC class IIB allele composition of these two groups (ANOSIM, $R_{\text{global}} = 0.001$, $P = 0.474$, average dissimilarity = 54.45%). However, the SIMPER test showed that there was one allele that accounted for 8.61% of the allelic difference between the two groups. The likelihood of possessing this allele increased with increasing allele numbers per individual [(GLM (binomial, logit link), $X^2_{1;191} = 21.293$, $P < 0.0001$].

For the entire reproductive period, the two food groups differed significantly in the reproductive investment per subperiod of individuals with and without that specific allele (repeated measurements MANOVA, time · food group · allele: $N = 75$, $F_{4,68} = 2.894$, $P = 0.028$, Fig. 4). Whereas there was an overall effect of time under low food conditions showing that the number of spawnings decreased rapidly (repeated measurements MANOVA, $N = 36$, $F_{4,31} = 21.912$, $P < 0.0001$, Fig. 4), we found no difference between individuals carrying the specific allele and those not carrying it (repeated measurements MANOVA, time · allele: $N = 36$, $F_{4,31} = 0.785$, $P = 0.544$, Fig. 4). However, individuals of the well-fed group differed significantly in their reproductive investment across the breeding period depending on the presence of the specific allele (repeated measurements MANOVA, time · allele: $N = 39$, $F_{4,34} = 4.057$, $P = 0.009$, Fig. 4). Individuals not carrying the allele reproduced early in the reproductive period followed by a decrease of spawning rate (repeated measurements MANOVA, $N = 25$, $F_{4,21} = 11.741$, $P < 0.0001$, Fig. 3b), whereas females that possessed the allele spawned continuously throughout the whole observation period (repeated measurements MANOVA, $N = 14$, $F_{4,10} = 0.625$, $P = 0.655$, Fig. 4).



Chapter I Fig. 3: Number of egg clutches spawned by female sticklebacks during the 25-day assessment time in relation to individual MHC class IIB allele diversity. Filled circles represent the well-fed, open circles the poorly fed group. Data points were spread for clarification.



Chapter I Fig. 4: Mean number of egg clutches spawned by female sticklebacks per reproductive subperiod. The 25-day assessment time was divided into five subperiods consisting of 5 days each. Females were grouped according to their food treatment and the possession of the identified specific MHC class IIB allele. The allele was found to be carried significantly more often by females that still spawned in the last subperiod. Error bars indicate one standard error.

Discussion

The observed mean individual MHC class IIB allele diversity of 5.6 is close to both the mean allelic diversity found in a natural population (Reusch et al. 2001a) and the optimal allele number found both here for body condition, i.e. 6.2, and in other studies (Aeschlimann et al. 2003; Milinski 2003; Wegner et al. 2003a; Wegner et al. 2003b; Kurtz et al. 2004). Also in this study, we found that individuals owning an optimal MHC class IIB number of about six alleles were in better condition than individuals carrying a nonoptimal allele number. The hypothesis of the two opposing selective forces acting on the number of MHC alleles per individual – increased pathogen recognition versus increased autoreactivity and thus increased deselection of T-cell clones (Nowak et al. 1992; De Boer & Perelson 1993; Borghans et al. 2003; Milinski 2006) – should also apply to MHC class I. We predicted an optimal individual allele diversity also for MHC I. We would expect individuals carrying the median number of alleles to be the most frequent type. Indeed we found that the median was the most frequent type and had seven alleles.

- **Male traits**

o **Nest quality**

We observed that males possessing an intermediate MHC class IIB allele diversity owned nests of higher quality than males with a non-intermediate allele number. This adds another trait to the list of characteristics which are of highest quality when individual MHC class IIB diversity is optimal, concurring with previous correlative and experimental studies that have also found an optimal individual diversity of about six MHC class IIB alleles in sticklebacks (Reusch et al. 2001a; Aeschlimann et al. 2003; Milinski 2003; Wegner et al. 2003a; Wegner et al. 2003b; Kurtz et al. 2004; Milinski et al. 2005; Kurtz et al. 2006; Wegner et al. 2006).

Nest quality was influenced by an interaction between parasite load and food group. This means that poorly fed fish had nests of lower quality with increasing parasite load when compared with well-fed fish. Maintaining a high-quality nest involves regular glueing, which is likely to require a higher glue production. Glue is produced in the males' kidneys, which were larger in males with high-quality nests (Barber et al. 2001b) and in well-fed males (Wootton 1994). The pronounced difference that we found between the two food groups clearly emphasizes the importance of diet on individual performance and suggests that feeding regime should be carefully considered when conducting laboratory experiments (see also (Barber & Arnott 2000; Candolin & Voigt 2001). The effect of the interaction of parasite load and food level on nest quality fits our prediction from life-history theory (Stearns 1992). Male sticklebacks might be able to compensate for parasitization under good food conditions,

whereas this might not be the case when energy availability is low. Hence, as a trait used by females to evaluate males, nest quality seems to reflect condition in a complex way.

- **Breeding coloration**

We did not find that males with an optimal MHC diversity had the brightest breeding coloration. Instead, we found a positive correlation between a male's redness and its MHC class I allele number. However, this is not necessarily in contradiction with optimal allele diversity if there is a specific allele whose possession outweighs the disadvantage of being above the optimal allele number by providing resistance to a new predominant pathogen. Indeed, we identified one MHC class I allele that was associated with a more intense breeding coloration. Various studies have found specific MHC alleles to be associated with resistance against certain pathogens (e.g. (Langefors et al. 2001; Grimholt et al. 2003; Bonneaud et al. 2006b; Milinski 2006) and in the stickleback, four MHC class IIB alleles were found that were each significantly related to the resistance against one specific macroparasite species (Wegner 2004).

One reason for males to be redder when carrying an advantageous MHC class I allele might be that they can afford to reallocate the available antioxidants to the carotenoid-based breeding ornamentation (Brush & Reisman 1965) instead of using them as radical scavengers. Male sticklebacks showing a higher MHC based resistance could have a lower demand for innate immune activity, thus be less prone to oxidative stress, and therefore not so much in need of radical scavengers (von Schantz et al. 1999; Kurtz et al. 2004; Kurtz et al. 2006). After exposure to parasites, three-spined sticklebacks did reduce their carotenoid-based red coloration (Milinski & Bakker, 1990) and individuals with an optimal number of MHC class IIB alleles showed the lowest respiratory burst reaction, an innate immune mechanism that generates reactive oxygen molecules to kill pathogens (Kurtz et al. 2004). Moreover, sticklebacks with a nonoptimal MHC class IIB diversity have higher levels of MHC gene expression (Wegner et al. 2006), which correlates with increased oxidative stress (Kurtz et al. 2006).

It has previously been shown that the male stickleback's red throat is an honest signal revealing a male's health (Milinski & Bakker 1990a) and resistance to parasite infection (Milinski & Bakker 1991). Our findings support the good-genes hypothesis of Hamilton & Zuk (1982), suggesting that a male's condition might be shaped by the genetic value of its immune system, revealed by the intensity of the red ornamentation. Females choosing the reddest male would then gain fitness benefits for their offspring in two ways: through high-quality parental care offered by a healthy mating partner and also by ensuring that the offspring are endowed with a good immune system. This concurs with the finding that offspring of brighter

stickleback males are more resistant to infection by the cestode *Schistocephalus solidus* (Barber et al. 2001a). Furthermore, when redness can be linked to resistance against presumably common pathogens, mate choice based on odor that reveals a male's MHC alleles may be improved by visual mate choice. As suggested by Aeschlimann *et al.* (2003) and Milinski (Milinski 2003; Milinski 2006), this might guarantee that not only the optimal MHC allele number but also the relevant specific MHC alleles are included in the optimum for the offspring. Consequently, males with an intermediate number of MHC class I alleles should also be more likely to possess the specific allele. However, this was not the case in our study. Possibly, the pathogen against which this allele presumably provides resistance was not one of the predominant parasite species in the stickleback population the year prior to our study, so that this allele could not be detected by a redder coloration and thus not be subjected to female choice. From this hypothesis, we would predict that such alleles would be found included in the optimum in the following year.

- **Female traits**

o **Number of egg clutches.**

Wootton (1994) and Fletcher & Wootton (1995) concluded that the number of clutches spawned per breeding season is dependent on the current food supply, whereas clutch and egg size are a function of female size at the start of the breeding season, thereby being only little affected by food availability. This would mean that food shortage occurring later would reduce the number of spawnings. We found not only that females under good food conditions spawned significantly more egg clutches than poorly fed females, but also a significant interaction between food level and individual MHC class IIB diversity on the number of egg clutches.

We found a conditional strategy to spawn either early or continuously under good food conditions depended on the possession of a specific MHC class IIB allele. Females that possessed the allele, probably providing resistance against a current parasite, spawned continuously throughout the whole observation period and would probably have continued beyond this time. The likelihood of possessing this allele increased with increasing allele numbers per individual. Hence, as suggested for the allele that correlated with intense breeding coloration in males, the parasite against which this allele presumably provides resistance, had not been predominant in the stickleback population in the previous year and was thus not included in the optimum.

As evidence from the field suggests (Wegner, 2004), MHC alleles are under negative frequency-dependent selection by parasites, and we expect that parasites abundant in one year will not be predominant in the following year. In conclusion, we predict the following scenario for negative frequency-dependent selection of MHC class I and IIB alleles by

pathogens in sticklebacks. Females try to select a male that: (1) offers through olfactory cues the number of different alleles that, in combination with the females' own alleles, achieve the immunogenetic optimum for the offspring; and (2) displays costly sexual signals which increase the probability that the complement from this male contains alleles that provide resistance against currently predominating parasites. We assume that the female assembles the optimal number of six alleles, which in the best case are composed of the alleles providing highest resistance against the currently predominating parasites. But how are advantageous MHC alleles of females selected for the offspring's optimum if the optimum is achieved by female choice? This study suggests that females with such alleles can achieve a higher reproductive output. Thus the 'good genes' of resistance of such successful females are probably ending up in the optimum that these females choose for their offspring. Thanks to the higher reproductive success of their mothers; these offspring will become numerous. Therefore, the previously dominating pathogens have only low prevalence in the following period of time. Now, other pathogens are predominating against which, e.g. any of the previous 'top six' MHC class IIB alleles no longer provide best resistance. Hence, other MHC alleles probably not contained in the previous optimum are providing highest resistance. The more alleles a fish carries, the more likely is this fish to carry the best resistance alleles just by chance, as suggested by this study. These alleles are then predicted to be part of the optimum of the next generation. This scenario awaits testing in a natural population during several generations. It opens the door to study the role of negative frequency-dependent selection in maintaining MHC polymorphism, and thus mate choice and sexual reproduction.

Acknowledgments

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Chapter II

Lifetime reproductive success is maximised with optimal MHC diversity

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Abstract

Individual diversity at the Major Histocompatibility Complex (MHC) is predicted to be optimal at intermediate rather than maximal levels. The advantage of immunogenetic recognition of different parasite derived antigens is traded-off against disadvantages of an increased loss of T cells from the repertoire through negative thymic selection. We showed previously in sticklebacks that an intermediate MHC diversity is predominant in natural populations and provides maximal resistance in experimental multiple parasite infections in the lab. However, what counts ultimately is life-time reproductive success (LRS). Here we measured LRS of six laboratory-bred sib-groups - to minimize the influence of non-MHC genes - three-spined sticklebacks during their entire breeding period, each in a semi-natural enclosure in the lake of their parents, where they were exposed to the natural spectrum of parasites. We collected developing clutches at regular intervals and determined parenthood for a representative number of eggs (2279 in total) per clutch with 18 microsatellites. Both males and females with an intermediate MHC class IIB variant number had the highest LRS. The mechanistic link of MHC diversity and LRS differed between the sexes: in females we found evidence for a trade-off between number of eggs and immunocompetence, whereas in males this correlation was concealed by different timing strategies of reproduction.

Introduction

Genes of the major histocompatibility complex (MHC) are the most polymorphic genes and play a fundamental role in the adaptive immunity of all jawed vertebrates. They encode cell-surface proteins, which present either self-peptides or peptides derived from phagocytosed pathogens to T lymphocytes, a prerequisite for production of pathogen-specific antibodies and the development of an immunological memory (Janeway et al. 2005). The enormous allelic diversity found in natural populations is generally regarded as a consequence of parasite-mediated balancing selection (Clarke & Kirby 1966; Takahata & Nei 1990; Apanius et al. 1997; Edwards & Hedrick 1998; Jordan & Bruford 1998; Penn & Potts 1999; Penn et al. 2002; Bernatchez & Landry 2003; Mays & Hill 2004; Milinski 2006). Several reports have shown correlations between certain MHC genotypes and occurrence or severity of specific diseases and parasite infections (Briles et al. 1983; Hill et al. 1991; Godot et al. 2000; Langefors et al. 2001; Grimholt et al. 2003; Bonneaud et al. 2005; Harf & Sommer 2005; Bonneaud et al. 2006b). This implies that individual MHC diversity should be maximised in order to achieve resistance against as many different pathogens as possible. However, although some studies show that MHC heterozygotes are more resistant than homozygotes (Doherty & Zinkernagel 1975; McClelland et al. 2003), the overall evidence remains ambiguous especially for species harbouring recently duplicated MHC loci (Penn 2002; Penn et al. 2002; Pitcher & Neff 2006). A recent study showed that mice that were heterozygous at all MHC loci were even less resistant than mice that were homozygous at all loci when challenged with different strains of salmonella (Ilmonen et al. 2007). Therefore, it seems to be disadvantageous to have too many different MHC alleles. Indeed, each time a distinct MHC molecule is added to the individual MHC repertoire, all T-cell clones that can recognise self-peptides bound to that molecule must be removed in order to maintain self-tolerance. This process of negative T-cell selection can prevent an efficient adaptive immune response if an individual has too many different MHC alleles (Lawlor et al. 1990). Thus, theoretical models predict that maximal pathogen resistance is achieved by an intermediate, i.e. optimal, rather than a maximal number of different MHC variants (Nowak et al. 1992; De Boer & Perelson 1993; Borghans et al. 2003; Milinski 2006; Woelfing et al. 2009). The most recent mathematical model (Woelfing et al. 2009), that is based on novel findings on T-cell selection, can predict the natural range of intra-individual MHC diversity. This prediction is in line with empirical studies, showing the lowest effect of parasite infections, a fitness trait, in fish (Wegner et al. 2003a; Wegner et al. 2003b; Wegner et al. 2008), and reptiles (Madsen & Ujvari 2006) with intermediate MHC diversity. Such a beneficial amount of individual MHC diversity may not only be maintained by parasite-mediated selection, but also amplified by MHC-based mate choice. For instance, female sticklebacks prefer males with a complementary number of MHC class II variants to their own

set of variants, resulting on average in intermediate immunogenetic diversity in their offspring (Reusch et al. 2001a; Aeschlimann et al. 2003; Milinski et al. 2005). Recently, Forsberg *et al.* (2007) found evidence for a similar mating preference of female brown trout (*Salmo trutta*) for males with intermediate MHC dissimilarity. Thus, fitness should ultimately, i.e. during an individual's lifetime, be maximised when MHC allele diversity is intermediate.

In this study, we tested the effects of individual MHC class II diversity on lifetime fitness by measuring the lifetime reproductive success (LRS) of three-spined sticklebacks (*Gasterosteus aculeatus*) under natural conditions. Sticklebacks offer an exceptional system because in populations of our geographical latitude, they usually have only one reproductive period in their life (Wootton 1976). Moreover, life history traits involved in reproduction have been studied extensively, such as breeding coloration that has been shown to be a good predictor of parasitism and body condition (Milinski & Bakker 1990b; Frischknecht 1993; Folstad et al. 1994; Kraak et al. 1999; Barber et al. 2000). In a field enclosure system, we analysed mating combinations and individual reproductive output of laboratory-bred sticklebacks. Within each of six enclosures, fish (8 males and 8 females) were full siblings, to reduce the influence of non-MHC genes (genetic background) and fish differed almost only with respect to their MHC genotype. Experimental fish had been individually challenged twice with three common sympatric macroparasites from the lake of their parents to simulate a natural life history comparable to their free-living conspecifics. In the enclosures we collected all developing clutches of eggs until the end of the reproductive season and allocated the most likely parents to each clutch. Therefore, using genetic methods we could estimate the LRS of each individual fish. Immunogenetic optimality should ultimately contribute to LRS. Therefore, we expect that fish with an intermediate MHC class IIB diversity have the highest reproductive success. This would explain why individuals with an intermediate MHC diversity predominate natural populations (Reusch et al. 2001a).

Materials and Methods

Experimental fish. Three-spined sticklebacks caught from a natural population from the lake Großer Plöner See were used for breeding as described elsewhere (Kalbe & Kurtz 2006). Several sibships per breeding pair were raised until the age of three months when offspring from the same pair were combined and transferred to 190L tanks in densities of 100 to 250 fish. Fourteen randomly chosen individuals and both parents of each sibship were analysed for their MHC class IIB genotype (see below). Six sibships with segregating numbers of MHC class IIB alleles/variants were selected, as in Wegner *et al.* (2003a). In such families, with a similar genetic background, MHC genotypes with different numbers of sequence variants were

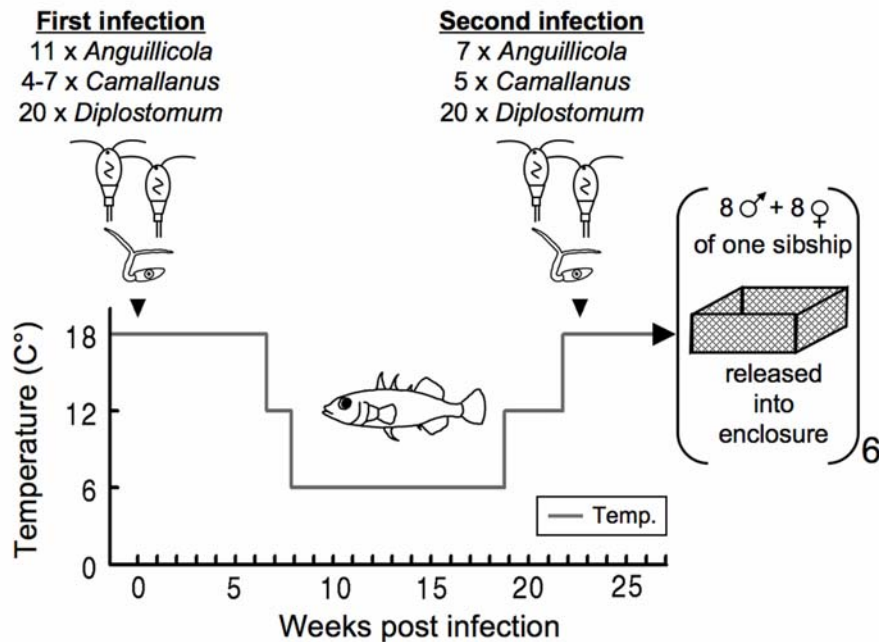
present: 1) intermediate diversity (around 6 sequence variants), a low number (< 5 sequence variants) and a high (> 7 sequence variants).

Experimental parasite infections and time schedule. From each sibship 30 randomly selected fish were each experimentally exposed twice to a combination of three of the most prevalent macroparasite species originating from their parents' habitat: the nematodes *Anguillicoloides crassus* and *Camallanus lacustris*, as well as a digenean trematode, the eye fluke *Diplostomum pseudospathaceum*. All parasites originated from the Großer Plöner See or contiguous neighbouring lakes and, therefore, are regarded as sympatric to the stickleback population examined here. Infection of fish was performed as described elsewhere (Kalbe & Kurtz 2006; Krobbach et al. 2007). Fish were exposed to the combination of all three parasites in December 2004, and again in May 2005. Between treatments, to mimic natural life history including parasite exposure, sticklebacks were stepwise brought to laboratory winter conditions (6°C, 10 h light per day) before re-experiencing summer conditions (18°C, 16 h light per day, see Fig. 1 for infection dosages and schedule).

Enclosures. The outdoor experiment was conducted in the lake Großer Plöner See, in northern Germany (54° 9'21.16"N, 10°25'50.14"E) during the summer 2005. The enclosure system consisted of six stainless steel mesh cages (3 m x 3 m, total height 1 m, 0.4 – 0.6 m above water surface) installed on the lake ground in a row close to the shoreline, located in the natural breeding area, i.e. where the parents of the experimental fish had been in the previous year. The 5 mm meshes allowed only small particles and most invertebrates (food items and intermediate hosts of various parasite species) to pass through. A coarse meshed net protected the fish from bird predation. Prior to release into the enclosures, we weighed (+/- 0.1mg), measured (+/- 1mm) the fish and counted the *Diplostomum* metacercariae in each eye lens under a dissection microscope. Finally, a 2-3 mm piece of the first dorsal spine was cut for genotyping at the end of the experiment. Each of the six enclosures was stocked with eight males and eight females, one family per cage, on June 16, 2005.

Egg collection. Every week but one, because the weather did not allow it, all stickleback nests were detected in each enclosure by careful observation. All egg batches were removed and the nests carefully replaced in their original location. Egg batches were brought to the laboratory; clutches were separated on the basis of different developmental stages. If necessary, the individual egg clutches were incubated in aerated well water (with 0.04 ppm malachite green) at 18°C until dark eye spots and the neural tube developed, to ensure a sufficient amount of DNA for further analysis (see below).

Recapture and examination of sticklebacks. During the last egg collection on August 3, when nests no longer contained fresh clutches, all surviving fish were caught. Immediately following capture, a picture of each male's red throat was taken within a dark box using a digital camera (Olympus E20-p) with a 36 mm macro-lens. For the camera parameters and the handling of fish, see Jäger *et al.* (2007). Intensity analysis of the red coloration was performed with IP Lab 3.6.2 for Mac OS 9.2.2 (Scanalytics, Inc.) delimitating a defined area of the red throat. Jäger *et al.* (2007) showed that method to be highly repeatable.



Chapter II Fig 1: Schematic representation of experimental infection schedule and temperature regime in the laboratory, prior to release of the sticklebacks into the enclosure.

Surviving fish were measured, weighed and dissected within three days after capture, including screening for macroparasites and weighting of organs (gonads, kidney, liver, spleen). General body condition of the fish was calculated according to Bolger & Connolly (1989).

All external and internal macroparasites and ciliates were determined to the lowest taxonomic level possible. In order to quantify the total parasite load of each fish, an individual parasite index was calculated (Kalbe *et al.* 2002). This allowed for summarising different combinations of rare and frequently occurring parasites for each stickleback and comparing their total parasite burden quantitatively.

MHC and microsatellite typing. DNA extractions from dorsal spines of fish before release in the enclosures and after recapturing, as well as from developing eggs, were conducted using DNA Tissue kit (Invitex, Germany) following the manufacturer's protocol. All the fish and a representative subsample of the collected fry (see below) were typed for 18 microsatellites

(see- Appendix II) combined in 5 different PCR protocols (Largiadèr et al. 1999; Peichel et al. 2001). This number of microsatellites was needed in order to guarantee a high parenthood resolution even within sibships. At the end of the experiment, survivors were re-typed for identification. The MHC class IIB diversity was determined using capillary electrophoresis single-strand conformation polymorphism (CE-SSCP) of the amplified exon 2 of the MHC class II β chain as described in Binz *et al.* (2001) (see Reusch & Langefors (2005) for estimation of the number loci).

Parenthood analysis and heterozygosity. From each egg clutch, 16 eggs were randomly picked for parentage analysis. Paternity assignments were performed with the software PAPA for every egg (Duchesne et al. 2002). Accordingly, each egg was assigned to one of the following male categories: nest owner, sneaker and stolen. Males that fertilised the majority of the eggs in a nest were categorised as nest owners and these eggs were assumed to originate from females that have chosen this male. Eggs were assigned to be fertilised by a sneaker, if in one nest the same female had eggs fertilised by the nest owner and another male. Eggs were assigned to be stolen from other nests if the nest owner did not fertilise them and no further eggs of the same female were found in the nest. Moreover, we verified that the combination existed in other nests to exclude wrong affiliations from the software. We also calculated an heterozygosity index for each individual parent (Coulson et al. 1998).

Data analysis. All statistical analyses were conducted in JMP 5.0.1 (SAS Institute). To start, a large multiple correlation was performed and occurring co-linearities were corrected by taking the residuals of the regressions. Stepwise model selections was performed based on the AIC criterion (Sakamoto et al. 1986).

LRS was calculated for both surviving and total number of fish since some dead fish had a certain fertilisation success.

- **Lifetime reproductive success (LRS).**

We aimed at estimating the lifetime reproductive success (LRS) of each fish. We performed an ANCOVA on the total number of eggs assigned to each parent, with enclosures, sex, parasite load, initial and final body condition (expressed as residuals of the regression with initial body condition) as dependent variables. Secondly, we correlated the residuals of the previous model for LRS with MHC class IIB diversity with both linear and quadratic terms.

With our design we had to test the correlation between MHC genotypes and LRS within sibships. We used Wegner *et al.*'s method (2003b) extended from Aeschlimann *et al.* (2003) and (Milinski 2003) to calculate reproductive success dependent on the number of individual MHC variants using each sibship as a statistical unit. Briefly, we averaged the number of eggs

within each sibship over each MHC genotype and calculated all the possible slopes between these points. For a first “purist” test we used the average number of eggs and the average slope derived as described and used this as one pair of data points from each sibship (see Results section for detailed description of analysis and the appendix II for a fictitious example explaining this method). Further tests took individual fish and thus more information into account. Because we had clear predictions with intermediate genotypes to perform better, we used directed (not one tailed) statistical tests (Rice & Gaines 1994). This analysis was performed with all introduced fish as well as only with the survivors.

- **Parasite load, body condition, spleno-somatic index and coloration.**

Surviving fish were tested for significant effects on parasite load with an ANCOVA incorporating body condition at the beginning of the experiment, body condition at the end, sex and enclosure as predictors. We also included all two-way interactions in the model. Thereafter, we correlated the residuals with MHC individual diversity.

As an estimate of the sticklebacks’ immunological activation, we calculated a spleno-somatic index (SSI) as followed: $SSI = (\text{spleen weight/body weight}) * 100$. We performed an ANOVA with SSI as dependent variable and enclosure, sex and their interaction as predictors. We then correlated the residuals with individual MHC diversity.

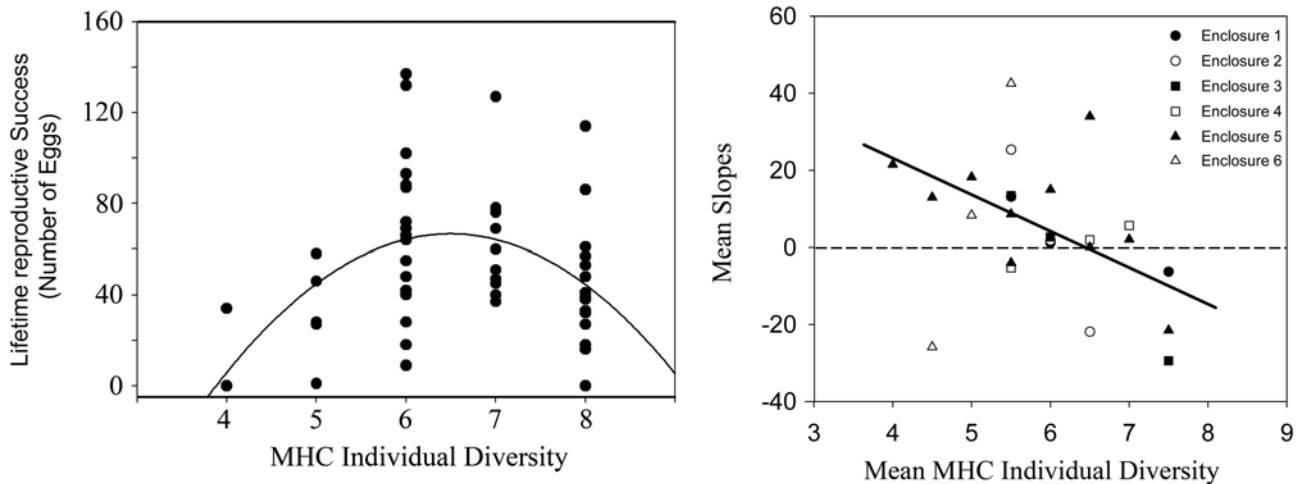
For the male breeding coloration, after an ANCOVA was performed to test for the effect of enclosures, body condition at the beginning and at the end of the experiment and of parasite load, we related the residuals of that model to individual MHC diversity.

Results

Lifetime reproductive success (LRS). Over the entire breeding we collected a total of 149 egg clutches until after 7 weeks the nests no longer contained fresh clutches. Using 18 microsatellite loci, 2279 out of the total of 2384 (95.6%) offspring could be unambiguously allocated to a pair of parents. The remaining offspring were removed from subsequent analyses. One percent of the eggs were evidently stolen from other nests, whereas sneaking behaviour accounted for 12% of eggs. Hence, although female sticklebacks tend to avoid inbreeding (Frommen & Bakker 2006), but had no choice here, the sneaking rate of males within families in the enclosures was in the same range as in natural populations (Rico et al. 1992; Largiadèr et al. 2001; Blais et al. 2004). The total number of analysed offspring varied between 1 and 137 eggs per individual fish. The lifetime reproductive success (LRS) of all fish released in the enclosures was significantly correlated only to MHC diversity: fish with an intermediate number of MHC alleles had the highest LRS (Fig. 2a; $N=96$, $t=-2.40$, $P=0.0185$, table1, a & a1). None of our other variables could predict LRS (Table1, a). This result

remained significant when restricted to surviving fish recaptured at the end of the experiment (N=53, $t=-2.55$, $P=0.014$, table1, a2) and when recaptured males and females (from 48 of each sex originally introduced) were considered separately (female, N=28, $t=-2.94$, $P=0.007$; males, N=25, $t=-2.46$, $P=0.022$). Furthermore, LRS was not related to our individual heterozygosity index based on neutral markers (Linear: $F_{1,53}=0.155$, $P=0.695$; Quadratic: $F_{2,53}=0.207$, $P=0.137$).

Comparing LRS among sibships. if a genotype with an optimal number of MHC alleles has the highest number of eggs, the slope between egg number of this genotype and that of a genotype having less alleles should be positive, whereas it should be negative between the optimal genotype and a genotype with more alleles. Moreover, two genotypes equally distant from the optimum should have the same egg number and thus a slope of 0. Furthermore, the mean of the slopes should be positive when the mean of allele numbers is lower than the optimal number and vice versa. If there is an optimal MHC allele number per individual, all single sibship data points (relation between mean slope and mean MHC variant number of a sibship) should fall on one straight line with negative slope with the x-axis intercept at the hypothesised optimum (see Wegner *et al.* (2003a) for a similar analysis and the appendix II for a fictitious example explaining this method). Indeed, we could detect a significant negative linear relationship between the mean slopes of LRS and the mean number of MHC class IIB sequence variants (all fish averaged per sibship, directed test $F_{1,5}=5.44$, $P=0.05$; surviving fish, directed test $F_{1,5}=7.28$, $P=0.034$). This analysis treated each sibship as a statistical unit. With fish averaged for each MHC individual diversity treated as statistical unit we found similar results (all fish, directed test $F_{1,23}=4.28$, $P=0.031$; surviving fish, directed test $F_{1,16}=19.28$, $P=0.0001$, see Fig. 2b). In the first case, the analysis resulted in an optimum at 6.55 MHC variants while it resulted in an optimum of 6.40 when considering the surviving fish only. Males had a higher variance in their LRS than females (mean +/- SE, males: 55.4 +/-7.92 eggs; females: 53.11 +/- 4.57 eggs, Levene test, $F_{1,52}=5.495$, $P=0.023$).



Chapter II Fig. 2:

a) Relationship between lifetime reproductive success (shown as representative number of eggs) and MHC class IIB diversity. Fish with an intermediate MHC II B diversity had the highest reproductive output ($N_{\text{eggs}} = -0.272 + 0.136N_{\text{MHCIIIB}} - 0.499(N_{\text{MHCIIIB}} - 6.679)^2$, $F_{2,52} = 4.29$, $P = 0.019$). For clarity, raw data of surviving fish are presented but tests were performed on residuals as stated in Materials and Methods section. b) Relationship between mean slopes of all potential pairs of surviving fish within an enclosure and their relative mean MHC diversity. All data points obtained from the same enclosure are depicted by the same symbol. The equation of the linear regression is $f(x) = -27.94x + 178.81$, directed test $F_{1,17} = 33.71$, $P = 0.0001$.

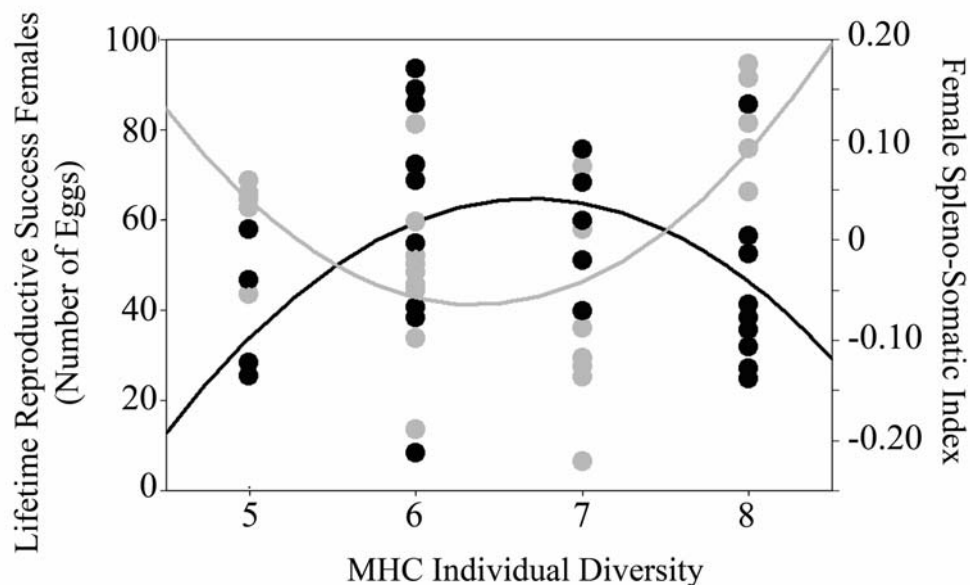
Spleen size. The spleno-somatic index (SSI), an indicator for immune activation, was different among enclosures ($F_{5,51} = 7.96$, $p < 0.0001$), and between the sexes with females showing higher values ($F_{1,51} = 6.87$, $P = 0.012$). Due to sex differences we split the data set and correlated SSI with individual MHC diversity for each sex separately. Male SSI did not show a significant relationship with MHC diversity (linear, $t_{1,25} = -1.30$, $P = 0.206$; quadratic, $t_{1,25} = -1.93$, $P = 0.083$). Females with an intermediate number of MHC variants had the lowest SSI (Fig. 3; quadratic, $t_{1,28} = 3.41$, $P = 0.002$; linear, $t_{1,28} = 1.28$, $P = 0.212$. table 1b). Moreover, SSI correlated with the total egg number per individual for females (directed test, $t_{1,28} = -1.84$, $P = 0.049$) but not for males (directed test, $t_{1,25} = 0.88$, $P = 0.242$).

Parasite load. We recorded 21 different parasites (for details see Appendix II) from 8 taxonomic groups: Protozoa (*Trichodina* sp., *Apiosoma* sp. and *Ichthyophthirius multifiliis*), Monogenea (*Gyrodactylus* sp.), Digenea (*Diplostomum* sp., *Apatemon cobitidis*, *Phyllodistomum folium*, *Cyathocotyle prussica*, *Tylodelphis clavata*, *Echinochasmus* sp.), Cestoda (*Valipora campylancristrota*, *Proteocephalus filicollis*), Nematoda (*Anguillicoloides crassus*, *Camallanus lacustris*, *Contracaecum* sp., *Raphidascaris acus*), Acanthocephala

(*Acanthocephalus lucii* and *A. clavula*), the crustaceans *Argulus foliaceus* and *Ergasilus* sp. and glochidia, the parasitic larval stages of freshwater mussels (Mollusca).

The total parasite load (table 1c) varied significantly among the enclosures ($F_{5,53}=4.145$, $P=0.004$), and females were more infected than males ($F_{1,53}=13.123$, $P<0.001$). Infection intensities by trophically transmitted parasites (all nematodes, cestodes and acanthocephalans found in this study) were higher in females than males ($t_{1,52}=3.050$, $P=0.004$), whereas there was no difference for directly and actively transmitted parasites (active, $t_{1,52}=1.291$, $P=0.203$; direct, Mann-Whitney test, $Z=0.632$, $P=0.527$, see Scharsack *et al.* (2007).

Only for the eye fluke *Diplostomum* sp., but not for the two internal nematode parasites, we could determine the effect of the laboratory infection *in vivo* prior to release into cages. Here, parasite burden increased in the enclosure period significantly (paired t-test on log transformed data, $t_{1,52}= 15.994$, $P<0.001$) with fish that harboured the highest number of *Diplostomum* sp. at the beginning also showing the highest *Diplostomum* sp. intensity at the end ($R^2=0.18$, $F=10.414$, $P=0.002$).



Chapter II Fig. 3: Graph combining female lifetime reproductive success (shown as representative number of eggs) and spleno-somatic index (SSI) as function of MHC II B diversity. In black, females with an intermediate MHC II B diversity had the highest reproductive success ($N_{\text{eggs}}=38.38+4.514 N_{\text{MHCII B}}-11.98(N_{\text{MHCII B}}-6.481)^2$, $F_{2,26}=4.63$, $P=0.02$). In grey, females with an intermediate MHC II B diversity had the lowest SSI, representing a better immuno-competence status ($\text{SSI}=-0.193+0.02 N_{\text{MHCII B}}+0.057(N_{\text{MHCII B}}-6.536)^2$, $F_{2,26}=6.698$, $P=0.004$).

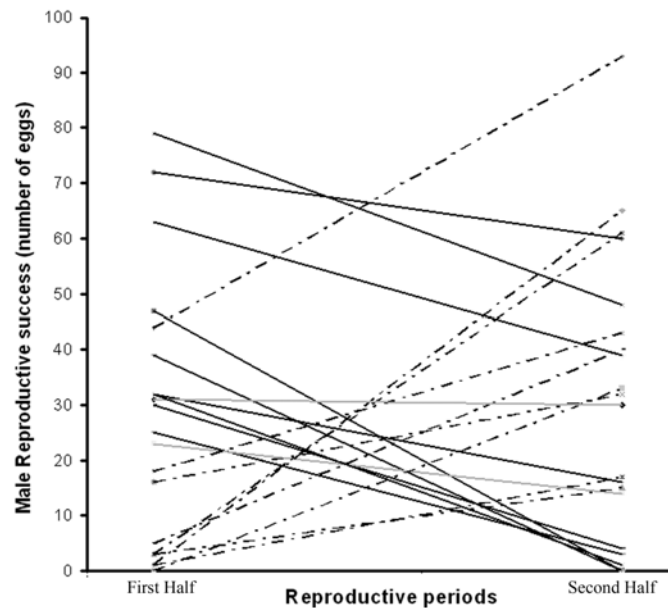
Body condition and coloration. Body condition, a function of a species specific combination of body length and weight (Frischknecht 1993), was estimated at the beginning and at the end of the experiment for both sexes. The loss in body condition was positively correlated only to parasite burden ($F_{1,52}=9.15$, $P=0.004$) but not to MHC individual diversity (linear, $F_{1,52}=0.001$, $P=0.979$; quadratic, $F_{1,52}=0.532$, $P=0.469$).

Mature male sticklebacks develop distinctive red coloration of the throat, as a secondary sexual character. The intensity of breeding coloration (Table 1d) was negatively correlated with parasite load ($F_{1,25}=4.997$, $P=0.0364$) but increased with a higher body condition ($F_{1,25}=4.678$, $P=0.0422$, see appendix II). We did not find any significant differences among families ($F_{5,25}=0.492$, $P=0.778$), and the intensity of coloration of males did not predict LRS ($F_{1,25}=0.408$, $P=0.531$). Finally, we found no significant correlation between breeding coloration and individual MHC diversity (linear, $F_{1,25}=0.416$, $P=0.526$; quadratic, $F_{2,25}=1.00$, $P=0.384$).

Both body condition and coloration of males were measured at the end of the experiment when all fish had ceased reproduction. The missing correlation of either measure with LRS and thus with MHC diversity might result from different males being reproductive early and late in the season, as Bakker and Mundwiler (1994) found in a field study. Also in the present study males differed in the time course of their reproductive effort: some reproduced predominantly early and others late (Fig. 4). The latter had a significantly lower body condition at the end (Welch t test, $t=-2.151$, $p=0.0384$), which suggests that the late reproducing fish were the slightly weaker fish which could reproduce only after the stronger fish had finished as in Bakker and Mundwiler's (1994) study. However, neither the individual MHC diversity distributions (two-sample Kolmogorow-Smirnov test, $D=0.1618$, $p=0.9698$) nor the LRS (Welch t test, $t=0.939$, $p=0.3589$) differed significantly between the two groups.

Terms	Estimates	Standard error	t ratio	Prob>t	Terms	D.F	Sum of Squares	F ratio	Prob>F
(a1) Lifetime reproductive success- <i>Polynomial regression- Optimum</i>	0.055	2.907	0.02	0.985	(a) Lifetime reproductive success	5	5156.117	0.992	0.434
-MHC class II B	-4.378	1.826	-2.40	0.019*	-Sex	1	180.698	0.174	0.679
-MHC class II B (quadratic)					-Parasite load (corrected for enclosure and sex)	1	1189.776	1.142	0.291
(a2) Lifetime reproductive success surviving fish- <i>Polynomial regression- Optimum</i>	0.136	0.252	0.54	0.592	-Initial body condition	1	1175.581	1.129	0.291
-MHC class II B	-0.499	0.195	-2.55	0.014*	-Final body condition (corrected for parasite load and initial body condition)	1	0.006	0.00	0.998
-MHC class II B (quadratic)					(c) Colouration- <i>ANCOVA</i>				
(b) Spleno-Somatic Index - <i>Polynomial regression- Optimum</i>	0.02	0.0157	1.27	0.215	-Parasite load (corrected for parasite load and body condition)	1	207.987	4.997	0.0364*
-MHC class II B	0.0567	0.0169	3.36	0.0025**	-Body condition	1	194.716	4.678	0.0422*
-MHC class II B (quadratic)					(d) Parasite load- <i>ANCOVA</i>				
					-Enclosure	5	2.212	4.145	0.0036**
					-Sex	1	1.401	13.12	0.0008***
					-Body condition (corrected body condition and sex)	1	0.086	0.806	0.3742

Chapter II Table 1: Variables correlated with (a) lifetime reproductive success, (b) spleno-somatic index, (c) colouration, (d) parasite load. Models were chosen on the basis of AIC criteria



Chapter II Fig. 4: Graph showing the different male reproductive strategies during the experiment. Dashed black lines represent fish that significantly increased the reproductive success in the second half, while full black lines represent fish that decreased their activity in the second part. Grey lines symbolize fish for which there was no difference between the two periods.

Discussion

Several studies have now identified natural or sexual selection for intermediate rather than maximal MHC diversity (Reusch et al. 2001a; Aeschlimann et al. 2003; Wegner et al. 2003a; Wegner et al. 2003b; Bonneaud et al. 2004; Milinski et al. 2005; Madsen & Ujvari 2006; Forsberg et al. 2007; Wegner et al. 2008). Here, we show that an intermediate MHC diversity maximises lifetime reproductive success (LRS), i.e. Darwinian fitness. Our experimental design permitted the estimation of individual LRS because three-spined sticklebacks reproduce only during a single breeding season in their entire life (Wootton 1976). We found that LRS was highest in individuals with an intermediate MHC diversity, which corresponds to an immunogenetic optimum (Wegner et al. 2003a; Wegner et al. 2003b; Kurtz et al. 2004), even though infected by varied parasite communities in the different enclosures. While this result was significant for both males and females, the mechanistic link of MHC optimality to LRS might differ between the sexes. In our study body condition and parasite burden are directly linked to LRS probably only in females. Overall, females harboured more parasites than males. However, this does not necessarily indicate lower immunocompetence. The disparity is due only to differential infection intensities of trophically transmitted helminths, thus most likely reflecting different feeding behaviours of females to cover their higher energy demand. The

number of eggs a female stickleback can produce is dependent on its physiological capacity and nutritional status (Wootton 1977; Kraak & Bakker 1998). Hence, females need to consume more food (Wootton 1976), which also includes copepods and other potential intermediate hosts. Parasites with active transmission, like digenean trematodes, did not differ between the sexes in infection intensity.

In males, where the variance in LRS was higher than in females, the situation is more complex, probably because different males had different time courses in their reproductive effort (Fig. 4), as had been shown by Bakker and Mundwiler in a field study (1994), possibly indicating a terminal investment strategy of weaker fish. Therefore, neither breeding coloration nor body condition measured at the end of the experiment was correlated with LRS or individual MHC diversity. Because we could not take these measures repeatedly during the course of the experiment, we cannot detect any mechanistic link with individual MHC diversity. However, reproductive success was measured continuously, allowing us to show that males with an intermediate MHC variant number achieved the highest LRS.

Among condition dependent male traits in sticklebacks, the intensity of the red breeding coloration is one of the most conspicuous sexual traits that received a lot of attention (McLennan & McPhail 1990; Milinski & Bakker 1990a; Bakker & Milinski 1991; Milinski & Bakker 1992; Frischknecht 1993; Bakker & Mundwiler 1994; Candolin 1999; Kraak et al. 1999; Barber et al. 2000). Parasite infections affect breeding coloration (Milinski & Bakker 1990a; Folstad et al. 1994) and body condition (Milinski & Bakker 1990a; Tierney et al. 1996; Blais et al. 2004). Accordingly, as shown here, the coloration of males recaptured from the enclosures correlated positively with body condition but negatively with parasite burden measured at the end of the experiment. However, in accordance with previous studies, the quality of sexual ornaments was not greater in males with optimal MHC diversity, but rather could reveal the possession of specific MHC alleles (Buchholz et al. 2004; Jäger et al. 2007). These respective alleles probably provide resistance against the currently predominating parasite species (Jäger et al. 2007). Therefore, female mate choice is predicted to include two criteria: olfactory cues reveal a male's MHC variant diversity, whereas colour reveals the possession of currently protective alleles (Aeschlimann et al. 2003; Milinski 2006; Jäger et al. 2007). A trait correlating with individual MHC diversity in females was spleen size. Spleen size has been widely used in immuno-ecological studies as a measure of general activation of the immune system by multiple macroparasite infections, particularly in birds (John 1995; Moller & Erritzoe 1998; Morand & Poulin 2000; Brown & Brown 2002) and fish (Skarstein et al. 2001; Kortet et al. 2003; Lefebvre et al. 2004; Ottova et al. 2005). Females with an intermediate number of MHC class IIB variants had the lowest relative spleen size (Fig. 4), which correlated (marginally

significant) with the number of eggs assigned to the respective females. This potential link between reproduction, MHC genotype and spleen size is probably due to the activity of the immune system itself, rather than to the result of its efficiency, namely the individual parasite burden. This suggests a trade off between an individual's LRS and the costs of immunity. Sticklebacks with a more efficient adaptive (MHC-dependent) immune system, can afford to invest more into their offspring, whereas fish with less optimal MHC diversity need to allocate a higher proportion of their resources for defence mechanisms to maintain their parasite load at tolerable levels. Previous studies already showed that sticklebacks with an intermediate MHC diversity had the lowest oxidative burst activity, but were nevertheless more capable of limiting the growth of the tapeworm *Schistocephalus solidus* than fish with MHC variant number deviating from this optimum (Kurtz et al. 2004). Fish with an optimal MHC diversity basis seem to perform a shift from costly and self-damaging innate immune function towards a probably less costly, but efficient adaptive immune strategy, and use immunological mechanisms more concerted and economically. Costs of immunity are predominantly regarded as metabolic constraints (Lochmiller & Deerenberg 2000). The results of the present study indicate that immunological costs might directly affect LRS, but that their impact depends to a high degree on the individual MHC genotype. Therefore, these results may further explain why sticklebacks with intermediate MHC diversity prevail in natural stickleback populations (Reusch et al. 2001a; Wegner et al. 2003a).

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Chapter III

MHC-based mate choice combines good genes and maintenance of MHC polymorphism.

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Abstract

Polymorphic genes of the major histocompatibility complex (MHC) are regarded as essential genes for individual fitness under conditions of natural and sexual selection. To test this hypothesis, we investigated the ultimate individual fitness trait- that of reproductive success. We used three-spined sticklebacks (*Gasterosteus aculeatus*) in semi natural enclosures, located in natural breeding areas where the experimental fish had been caught. During their reproductive period, fish were exposed continuously to their natural sympatric parasites. By genotyping almost 4000 eggs with 9 microsatellites, we determined the parenthood and inferred female mating decision. We found that with reference to their own MHC profile, female sticklebacks preferred to mate with males sharing an intermediate MHC diversity. In addition, males with a specific MHC haplotype were bigger and better at fighting a common parasite (*Gyrodactylus* sp.) and were more likely to be chosen. This translated directly into Darwinian fitness since fish harboring this specific MHC haplotype had a higher reproductive output. We conclude that females based their mating decision on a specific MHC haplotype conferring resistance against a common parasite. This identifies and supports “good genes”. We argue that such interaction between host and parasite driving assortative mating is not only a pre-requisite to negative-frequency-dependent selection- a potential mechanism to explain MHC polymorphism, but also a potential source of speciation.

Introduction

Female mate choice commonly occurs in the animal kingdom (Andersson 1994). Females are expected to exert an active mate-choice if the benefits, e.g. direct or indirect, of such choosiness outweigh the costs. For instance, costs could include increased predation risk while seeking partners, whereas benefits could stem from direct gains such as paternal care or indirect advantages such as good genes (Andersson 1994). Good genes are genes that increase individual fitness (Andersson 1994). They can be transmitted to the next generation from mating with a male that bears an advantageous allele conferring higher body condition under specific environments. One could hypothesize that phenotypically recognizable and inheritable immune genes represent an example of good genes due to adaptation to the immediate pathogenic environment (Hamilton & Zuk 1982).

Recently, genes of the major histocompatibility complex (MHC) have received major attention across different fields (Yamazaki et al. 1976; Lawlor et al. 1990; Nowak et al. 1992; Schat et al. 1994; Parham & Ohta 1996; Janeway et al. 1999; Westerdahl 2004; Bonneaud et al. 2005; Milinski et al. 2005; Kurtz et al. 2006) as they appear to play a key role at the interface between natural and sexual selection (review in Milinski 2006).

MHC is a large cluster of genes involved in immune response and molecular self/non-self discrimination. MHC class I genes encode MHC molecules that are expressed on nearly every cell type. They serve invigilation against intra-cellular parasites, e.g. viruses, mostly by presenting antigens to cytotoxic CD 8+ T cells. MHC class II genes initiate defense against extra-cellular parasites: pathogen-derived peptides are presented to CD 4+ T helper cells to trigger a specific immune response (Klein 1986; Janeway et al. 1999). Hence, it is not surprising that several reports have shown correlations between certain MHC genotypes and the occurrence or severity of specific diseases and parasite infections (Briles et al. 1983; Hill et al. 1991; Godot et al. 2000; Langefors et al. 2001; Grimholt et al. 2003; Horton et al. 2004; Bonneaud et al. 2005; Harf & Sommer 2005).

Interest in MHC genes has expanded with evidence of its extreme polymorphism. The enormous allelic diversity found in natural populations is generally regarded as a consequence of parasite-mediated balancing selection (Clarke & Kirby 1966; Takahata & Nei 1990; Apanius et al. 1997; Edwards & Hedrick 1998; Jordan & Bruford 1998; Penn & Potts 1999; Penn et al. 2002; Bernatchez & Landry 2003; Mays & Hill 2004; Milinski 2006). Pathogen driven selection could act on MHC genes through heterozygote advantage, frequency dependent selection and/or habitat heterogeneity (Doherty & Zinkernagel 1975; Apanius et al. 1997; Penn & Potts 1999; Hedrick 2002; Ekblom et al. 2007).

Furthermore, MHC polymorphism has been suggested to be under sexual selection (Apanius et al. 1997; Edwards & Hedrick 1998; Milinski 2006). Indeed, several studies showed evidence for MHC-based mate choice (Potts et al. 1991; Wedekind et al. 1995; Penn & Potts 1999; Landry et al. 2001; Reusch et al. 2001a; Aeschlimann et al. 2003; Milinski et al. 2005; Olsson et al. 2005; Bonneaud et al. 2006a; Forsberg et al. 2007; Schwensow et al. 2008). Females are assumed to search for partners in order to acquire the best immunogenetic make up for their offspring, while they may also avoid mating with closely related males (Yamazaki et al. 1976; Potts et al. 1991; Brown & Eklund 1994; Penn & Potts 1998). In humans, mice and sticklebacks, olfactory based mate choice has been suggested to be MHC linked (Egid & Brown 1989; Ninomiya & Brown 1995; Wedekind et al. 1995; Reusch et al. 2001a; Aeschlimann et al. 2003; Milinski et al. 2005). Additionally, various secondary sexual traits, known to influence mate choice, have been correlated to MHC, e.g. the snood in turkey (Buchholz et al. 2004), and the red throat in stickleback (Jäger et al. 2007).

Combining analyses at neutral and adaptive genetic loci provides an efficient tool for evaluating the importance of various selective mechanisms, such as mate choice, on the evolution of immunogenetic competence. In contrast to MHC genotypes, which directly translate into phenotypes (Paterson et al. 1998; Langefors et al. 2001; Lohm et al. 2002; Wegner et al. 2003b; Bonneaud et al. 2006b), microsatellites are assumed to behave neutrally and variation is thought to be primarily driven by non-selective evolutionary factors such as mutations, gene flow or genetic drift (Nei 1987).

The three-spined stickleback (*Gasterosteus aculeatus*) is a perfect model organism to study evolutionary mate choice strategies. During the reproductive period, territorial males maintain a nest. The nest has been demonstrated to be central for MHC and male factor signaling (Milinski et al. 2005; Sommerfeld et al. 2008). Based on olfactory as well as visual cues, females make their mating decision and spawn in the nest. Before chasing the female away, the territorial male will fertilize the eggs unless a sneaker male steals the fertilization. After fertilization, the territorial male guards the eggs and adopts a costly fanning behavior over the eggs until they hatch (Wootton 1976). Males display an intense red breeding coloration that has been shown to indicate individual parasitization status (Milinski & Bakker 1990a), body condition (Bakker & Mundwiler 1994) and MHC genotype (Jäger et al. 2007). Dichotomic olfactory-based mate choice experiments demonstrated female to choose partners providing, in reference to her own MHC make up, an optimal number of MHC variants to the offspring (Reusch et al. 2001a; Aeschlimann et al. 2003; Milinski et al. 2005). Such female mate choice resulted in a higher reproductive success in lab-bred fish bearing an intermediate MHC diversity (Kalbe et al. submitted).

In the present study, we investigated female MHC-based mate choice of wild-caught three-spined stickleback through its reproductive period in a field enclosure system. The enclosures were stocked with a representative sub sample of the native population. These fish were therefore exposed to their natural spectrum of sympatric parasites, but protected from any kind of predatory pressure. Every week we collected egg clutches and genotyped a representative number of eggs per clutch with microsatellites. We could thus assign both parents and infer female mate choice (Kalbe et al. submitted). We predicted that individual fitness, i.e. individual reproductive success, would be correlated with MHC genotype and best parasite resistance. Under natural breeding conditions, 1) if heterozygote advantage would be the primary force driving female MHC-based mate choice, the more diverse males should be preferred over the less diverse, regardless of the females own MHC genotype. 2) If mate choice would favor production of optimal offspring with regard to MHC, we would expect females to seek mates providing complementary and compatible set of genes for the offspring. 3) Alternatively, if mate choice would favor locally adapted genes against the most prevalent pathogens, we would expect females to seek mates carrying specific MHC alleles. Under these scenarios, we aimed to identify whether MHC genes could be regarded as good genes. Finally, because females may be choosier when the number of available mates is higher, we manipulated male density. With this setup, we aimed to simulate better the natural conditions where females might have access to a high number of potential mates.

Material Methods

Experimental fish. Young of the year three-spined sticklebacks (*Gasterosteus aculeatus*) were caught on the 15th of December in the lake Großer Plöner See (54° 9'21.16"N, 10°25'50.14"E, Germany). They were kept in the laboratory under winter light and temperature conditions (8/24 light, 6°C) until the 3rd of May 2006 when they were brought to spring conditions (12/24 light, 12°C). On May 11, they were transferred to summer conditions (15/24 light, 18°C) before release on May 19 into enclosures located exactly in the area where the fish were caught (see for enclosure set up Kalbe et al. submitted). All fish were weighed, measured and spine clipped before release. The enclosures were stocked with 6 randomly allocated females and either 6 (sex ratio 1:1 in 3 enclosures) or 12 (sex-ratio 2:1, in 3 enclosures) randomly caught males. These enclosures have previously been shown to provide the conditions needed for natural mating behavior and acquiring the natural range of sympatric parasites but exclude predation pressure (Kalbe et al. submitted).

Egg collection. To estimate reproductive success and mate choice, once week, all stickleback nests were detected by careful observations. All egg batches were collected and the nests

carefully replaced in their original location. Egg batches were brought to the laboratory. Within a nest, clutches were separated on the basis of different developmental stages. When necessary, individual egg clutches were incubated in aerated well water (with 0.04 ppm malachite green) at 18°C, until dark eye spots and the neural tube developed, to ensure a sufficient amount of DNA for further analysis. DNA from 24 randomly picked eggs from each egg clutch was automatically extracted (Freedom evo robot, Tecan, Switzerland) using Invisorb® DNA Tissue HTS 96 kits (Invitex, Germany).

Male breeding coloration estimation and dissection. After 4 weeks in the enclosures, the experiment was stopped since the number of clutches collected decreased markedly (protocol as in Kalbe *et al.* (submitted)). During re-capture, males were caught above the nest they maintained. This allowed us to determine whether they sired all, a proportion, or none of the eggs contained in the nest on that collection day. This was then used to infer egg categories (see Material & Methods section parenthood analysis). The males' red throats were photographed in the field immediately after capture. The photographs were taken within a dark box, with a fixed light source, using a digital camera (Olympus E20-p) with a 36 mm macro-lens. For the camera parameters and the handling of fish, see (Jäger *et al.* 2007). IP Lab 3.6.2 for Mac OS 9.2.2 was used to estimate the red coloration intensity from a fixed area of the throat. Jäger *et al.* (2007) showed this method to be highly repeatable. The recaptured fish were measured, weighed and dissected within three days post re-capture. Dissection took place under a dissection microscope to determine external and internal macroparasites as well as ciliates harbored by the fish, to the lowest taxonomic level possible (Kalbe *et al.* 2002). In order to quantitatively compare parasite burden between fish, a parasite index was calculated summarizing different combinations of rare and frequently occurring parasites (Kalbe *et al.* 2002).

DNA extraction, microsatellites and MHC typing. DNA extractions, from dorsal spines, were performed using Invisorb® DNA Tissue kits (Invitex, Germany) following the manufacturer's protocol. All the fish were typed for 9 microsatellites multiplexed in 2 different PCR protocols both before release in the enclosures, and after recapture for identification (Kalbe *et al.* submitted). The number of microsatellites was sufficient to guarantee a high parenthood resolution. The MHC class IIB diversity was then determined by reference strand conformation analysis (RSCA) according to Lenz *et al.* (in prep), targeting the exon II of MHC genes, which encodes the highly variable peptide binding groove of the beta chain of the MHC molecules.

Parenthood analysis. Paternity assignments were performed for each representative egg with the software PAPA (Duchesne *et al.* 2002). According to parenthood analysis, each egg was

assigned to one of the following categories: nest owner, sneaker and stolen (Kalbe et al. submitted). Males that fertilized the majority of the eggs in a nest were categorized as nest owners and these eggs were assumed to originate from females mate choice. This was verified, for the last day, with the data from recapture where the males were caught maintaining their nests. Eggs were assigned to the sneaker category when, within a nest, the same female had eggs fertilized by the nest owner and another male. Eggs were considered as stolen from other nests if they were not fertilized by the nest owner and no further eggs from the same female were found in the nest (Kalbe et al. submitted).

Data analyses

- Female mate choice

In order to test whether females use MHC self-reference under natural conditions to chose their mates, MHC similarity between all females and all males from the same enclosure, was calculated as MHC variant-sharing values: the proportion of shared MHC variants in a pair is twice the number of the variants shared by two individuals, divided by the sum of the variants of each individual [$D=2F_{ab}/(F_a+F_b)$] (Wetton et al. 1987). For all females, taking into account the actual number of mates they had, using computer simulation we let them choose i) randomly between the males in their enclosures, ii) the most MHC dissimilar, and iii) the most MHC similar. We first focused on males we classified as having been chosen. We compared the observed distribution to the calculated ones. The first null hypothesis was that females selected mates randomly with respect to MHC variant-sharing. The second null hypothesis tested whether females aimed at increasing MHC diversity by seeking dissimilar males (heterozygote advantage). Finally, the third null hypothesis was that females favored MHC similar males to mate with (local adaptation). The randomization was run 1000 times per enclosure to generate null distributions. A Mann-Whitney test was used to compare the observed shared variant values with the randomly obtained values. Since all the analyses are performed enclosure-wise, we combined the results from the 6 enclosures using a binomial test. The same analyses were performed for males that obtained fertilizations only through sneaking behavior.

- Reproductive success, parasite load and male red breeding coloration

The statistical analyses were performed with JMP (5.0.1a, SAS Institute Inc.), R statistical package (v.2.5.0) and Primer v6 (Clarke & Gorley 2006). Normality and homoscedasticity were checked and statistics were conducted accordingly. If needed, variables were transformed to meet test assumptions. Variable co-linearities were removed using residuals. Moreover, because enclosure and male density effects were strongly correlated (Pearson $\chi^2=18.422$, $p=0.003$), only the male density variable corrected for the enclosure variable was included in

the analysis. Since male density was our only treatment, we included all possible double interactions with that predictor in the models. Model selections were performed stepwise and based on AIC criteria (Sakamoto et al. 1986).

We investigated the probabilities of breeding over the entire reproductive period using sex, male density, individual MHC diversity, standard length before the experiment, male density and all two-way interactions with male density as predictors in a binomial logistic regression. The dependent variable was coded as 1 for individual which reproduced and 0 for those which did not. An analysis of covariance (ANCOVA) was used to test whether individual reproductive success (square-root transformed total number of eggs) was related to predictors such as sex, male density, parasite burden, standard length before experiment, individual MHC diversity and all two-way interactions with male density. If analyses were sex-specific, we added the red throat coloration as new male predictor.

In another ANCOVA, individual parasite load (log transformed) was investigated in relation to sex, male density, standard length, individual MHC diversity and all two-way interactions with male density.

For each male, we calculated the arc-sin transformed proportion of sneaked eggs over the total number of eggs in nest that were fertilized by each male. An ANCOVA was then performed with reproductive success, standard length, male breeding coloration, individual MHC diversity, male density all two-way interactions with male density as predictors.

Eventually another analysis of covariance was used to test for variation in male red throat coloration. Individual MHC diversity, male density, parasite load, initial standard length and all interaction with male density were used as predictors.

Results

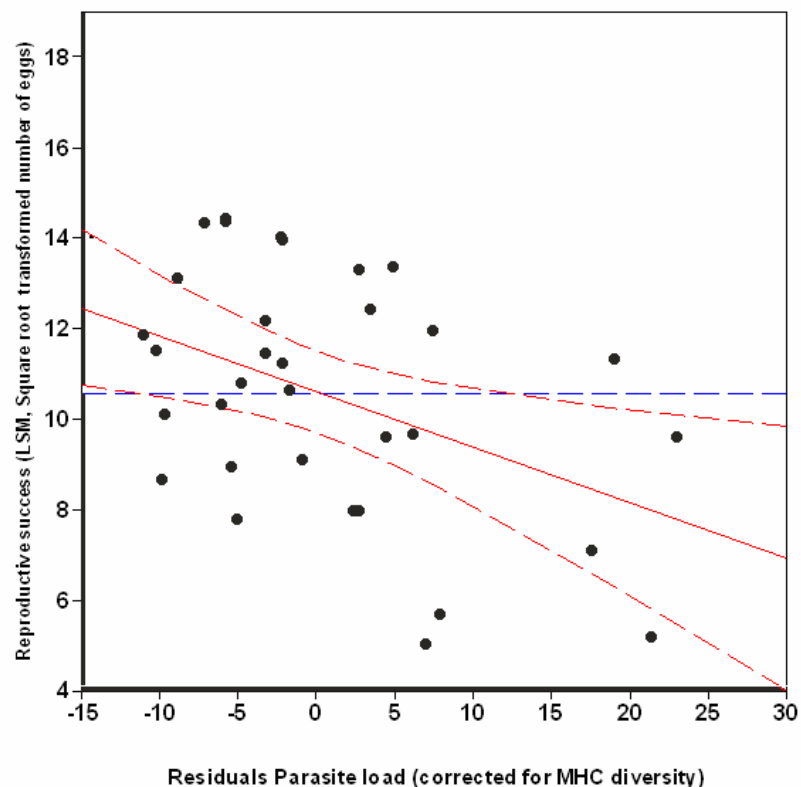
Recapture, parenthood analysis and MHC. After four weeks, 79 individuals out of 90 were recaptured (3 were found dead during the experiment) and 162 clutches were collected. Out of 3888 analyzed eggs, 32 (less than 1%) could not be assigned unambiguously to two parents and were therefore removed from further analyses. We found no deviation from Hardy-Weinberg equilibrium at any of the microsatellites in the experimental fish (All $p > 0.21$).

Transformations of the variable “individual MHC diversity” did not improve the data’s approximation to normal distribution. There was no relationship between the individual MHC diversity and sex (median=4, for both sexes. Mann-Whitney test, $U = 0.992$, $p = 0.321$). The number of variants ranged from 1 to 5 between individuals and the frequencies of the variants varied from 2 to 47%. There was no significant difference between enclosures, neither in MHC variant numbers (kruskal-wallis, $K = 3.384$, $d.f. = 5$, $p = 0.641$), nor in MHC variants constitution (ANOSIM on Bray-Curtis similarity matrix, Global $R = -0.039$, $p = 0.972$).

Female mate choice. Based on 9 microsatellites, females mate randomly with regard to genetic background (coefficient of relatedness from Queller & Goodnight, $r=-0.0119$, after 1000 bootstraps, $r=-0.0131$, $p>0.05$).

The probability of breeding during the experiment was positively related to fish length (binomial logistic regression, $X^2=5.797$, $p=0.016$), but not to individual MHC diversity (binomial logistic regression, $X^2=0.718$, $p=0.397$).

In each enclosure, females did not mate randomly with regard to MHC variant-sharing (Observed versus random distributions, Mann-Whitney tests for 6 enclosures, all $p<0.0001$. Binomial test for 6 enclosures, $p=0.0313$). The observed mating distribution was significantly different from the expected distribution if females would search for an MHC dissimilar partner (Observed versus maximum distributions, Mann-Whitney tests, all $p<0.0001$, binomial test for 6 enclosures, $p=0.0313$). Finally, females did not aim either to minimize MHC variant-sharing since the observed distribution was significantly different from the expected distribution in the case of seeking for MHC similar males (Observed versus minimum distributions, Mann-Whitney test, all $p<0.02$, binomial test for 6 enclosures, $p=0.0313$).



Chapter III Fig. 1: Figure representing individual reproductive success as a function of the parasite burden harbored by females ($F=7.458$, $p=0.011$). Blue dashed line shows the mean, the red dashed lines indicate the 95% confidence interval. The red line represents the regression line.

When investigating the number of MHC variants shared between females and males that gained fertilization by sneaking behavior, we could not detect any difference from random (Mann Withney tests, 3 enclosures $p > 0.05$, binomial test $p = 1$).

Individual reproductive success and fitness related traits

- Reproductive success

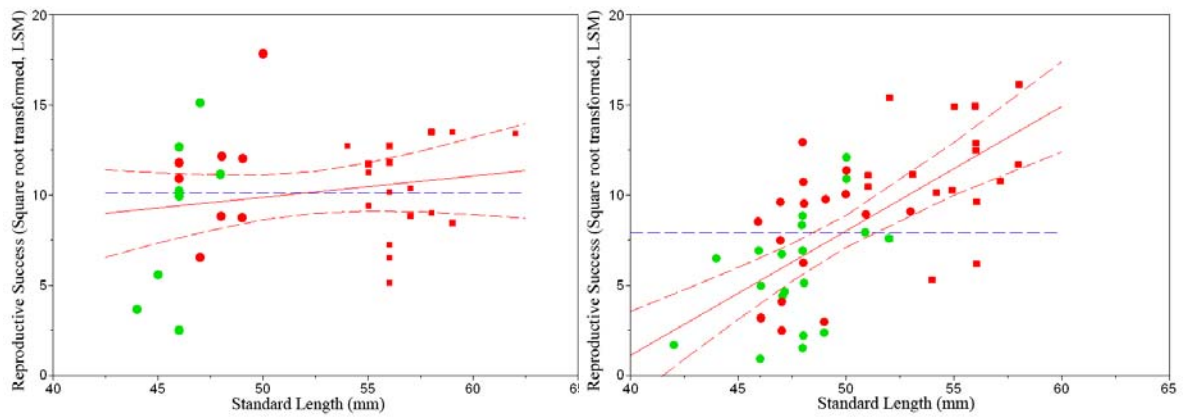
An ANCOVA revealed that larger fish, when corrected for sex variation, had a higher reproductive success than smaller ones ($F_{1,78} = 19.462$, $p < 0.0001$, Fig 2). Moreover, due to higher number of males reproductive success was strongly biased towards females ($F_{1,78} = 12.238$, $p < 0.0001$). Therefore we conducted the same analysis for each sex separately (Table 1). Female reproductive success was higher in enclosures with more males ($F_{1,30} = 4.336$, $p = 0.047$) and marginally with increased standard length ($F_{1,30} = 3.168$, $p = 0.086$). However, as parasite load increased, reproductive success decreased ($F_{1,30} = 7.458$, $p = 0.011$, Fig. 1; Table 1).

Contrary to females, male reproductive success was negatively affected by male density ($F_{1,46} = 18.105$, $p = 0.0001$). Nevertheless, male reproductive success positively correlated with standard length ($F_{1,46} = 24.224$, $p < 0.0001$) and negatively with parasite burden ($F_{1,46} = 4.489$, $p = 0.0399$). Surprisingly, the reproductive success was also negatively correlated with the male red coloration ($F_{1,46} = 6.172$, $p = 0.0170$; Table 1)

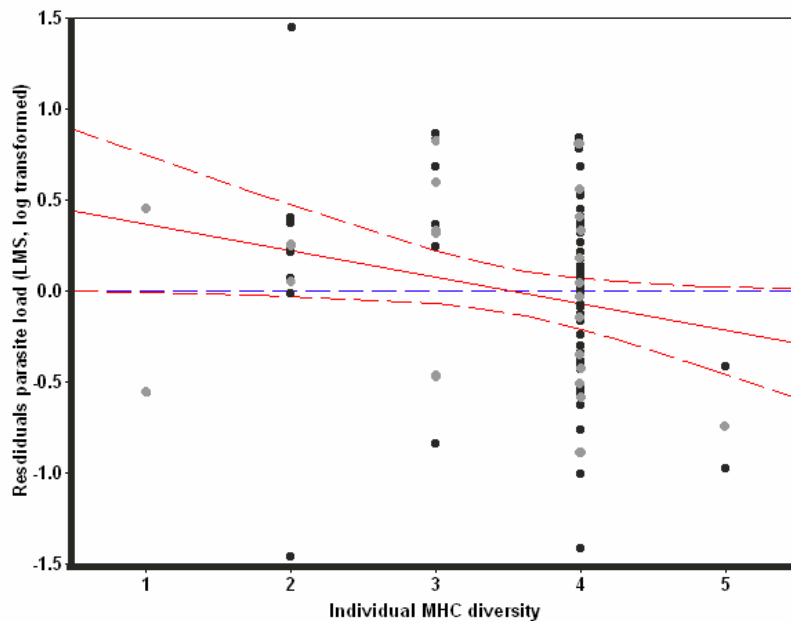
- Parasite load

Females harbored a higher individual parasite load than males ($F_{1,78} = 10.747$, $p = 0.0016$) and for both sexes, the parasite burden was negatively correlated to individual MHC diversity ($F_{1,78} = 4.730$, $p = 0.0328$, Table 2a, Fig. 3).

Since reproductive success was related to both standard length and parasite load, we investigated whether bigger and smaller fish harbored the same parasite communities. To assess this, we arbitrarily created three size groups for each sex: small, medium and large. An analysis of similarity on Bray-Curtis similarity matrix (ANOSIM) revealed that large and small males harbored different parasites (Males Global $R = 0.082$, $p = 0.026$. Females Global $R = 0.007$, $p = 0.32$). A similarity percentage-species contributions test (SIMPER) revealed that the tested fish groups were dissimilar for 43.14% and that 35.41% of that difference was explained by a lower parasitization by the monogenan *Gyrodactylus sp.* and the cestode *Valipora campylancristrota* in the bigger fish. Such relationship was detected only in males.



Chapter III Fig. 2: Figure showing the relationship between individual reproductive success as a function of standard length (Corrected for sex differences, $F=16.50$, $p<0.0001$). Blue dashed line show the mean, while the red dashed lines indicate the 95% confidence interval. The red line represents the regression line. Squares symbolize female, circles symbolize males, red is used for individuals with F10, green for individuals without F10. left) In low density enclosures, right) in high density enclosures.



Chapter III Fig. 3: Graph showing the relationship between individual parasite burden and MHC intra-individual diversity ($F=4.73$, $p=0.033$). Blue dashed line shows the mean, while the red dashed lines indicate the 95% confidence interval. The red line represents the regression line. Black dots correspond to fish with F10, while grey dot symbolize fish lacking F10.

We used the same statistical approach to investigate whether larger males had different MHC variants than smaller ones. And, indeed, the MHC genotypes of big males were significantly different from the MHC genotypes of smaller males (Global R=0.091, p=0.014). The average dissimilarity was 73.10% and was mainly due to the presence of the haplotype F10 (43.84%) in the bigger fish.

The standard length was higher for males with the MHC haplotype F10, and larger males harbored a lower burden of *Gyrodactylus* sp. and *Valipora campylancristrota*. We subsequently investigated whether these two parasites decreased when fish carried F10. Males with the MHC haplotype F10 (corrected for length) had a lower parasite burden in *Gyrodactylus* sp. than those without F10 (ANOSIM, Global R=0.084, p=0.047. Fig. 4). However, there was no difference for the cestode *Valipora campylancristrota* (ANOSIM, Global R=0.021, p=0.329). Note, the use of a permutation based test allows for no post-hoc correction.

Gyrodactylus sp. was one of the most prominent parasites. In total, its prevalence reached 67% and was consistent over all enclosures ($F_{5,79}=1.024$, p=0.409) and between sexes ($F_{1,79}=2.884$, p=0.093). Parasitized fish harbored from 1 to 244 *Gyrodactylus* sp. with a mean infection intensity of 13 individual parasites.

Chapter III Table 1: Reproductive success of the three-spined stickleback over one reproductive period. Reproductive success is given as number of eggs square-root transformed to meet normality. a) gives the best predictors for females, and b) gives the best predictors for males. NA stands for not applicable and concerns males' secondary sexual characteristics only.

a) Variables	FEMALES			MALES		
	d.f	F ratio	p-value	d.f	F ratio	p-value
Male density	1	4.336	0.0469	1	18.105	0.0001
Standard length	1	3.168	0.0863	1	24.224	<0.0001
Parasite load ^a	1	7.458	0.0110	1	4.489	0.0399
MHC diversity	1	0.122	0.7293	1	0.092	0.7637
Redness ^a	NA	NA	NA	1	6.172	0.0170
Standard length *density	1	0.008	0.9290	1	1.006	0.3216
Parasite load ^a *density	1	0.031	0.8607	1	0.045	0.9563
Redness ^a * density	NA	NA	NA	1	0.477	0.4934

^a corrected for MHC individual diversity

We investigated whether the haplotype F10 was advantageous over a broader array of parasites by replacing individual MHC diversity of our previous model, by the presence/absence of F10 (Table 2b). Here as well, male density and sex affected significantly the parasite load, but we did not detect any effect of F10 ($F_{1,78}=0.003$, $p=0.955$).

- **Re-analyses of female mate choice and reproductive success**

Since we found a correlation with a specific MHC haplotype, we investigated whether the probability of mating was a function of the presence or absence of that haplotype. We re-performed the same binomial logistic regression using F10 as a new variable (presence or absence of the haplotype). Again, we found that for both sexes the likelihood of mating during the reproductive period was positively correlated with fish length ($X^2=5.797$, $p=0.016$). Furthermore, fish with the haplotype F10 had a significantly higher likelihood of reproducing ($X^2=5.550$, $p=0.019$). Splitting the data set for sex showed that all females bared the haplotype F10, whereas 42.9% of the males harbored it.

Parasite burden, standard length of the fish and the probability of mating were dependent on whether the fish harbored the haplotype F10. These results led us to re-conduct the initial test investigating the reproductive success. Since all females displayed the haplotype F10, a female specific analysis was not possible and therefore our tests included all the fish (Table 3a) and only males (Table 3b). Interestingly, we found a positive role for both fish size and haplotype F10 on the reproductive output of the fish (ANCOVA, Size, $F_{1,78}= 24.259$, $p<0.0001$; Haplotype F10, $F_{1,78}= 4.596$, $p=0.035$, Fig. 5).

Chapter III Table 2: Results obtained from an analysis of covariance on log transformed individual parasite load a) with individual MHC diversity and b) with F10 as variable.

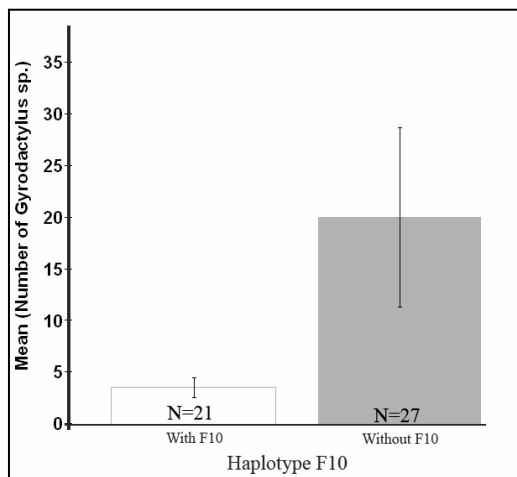
a) Variables	ALL FISH/ PARASITE LOAD			ALL FISH/ PARASITE LOAD		
	d.f	F ratio	p-value	d.f	F ratio	p-value
Male density	1	4.883	0.0301	1	4.015	0.0487
Standard length ^{a,b}	1	0.521	0.4728	1	0.785	0.3786
Individual MHC diversity	1	4.730	0.0328	NA	NA	NA
F10	NA	NA	NA	1	0.003	0.9545
Sex	1	10.324	0.0019	1	9.364	0.0031
Standard length ^{a,b} *density	1	1.379	0.2582	1	1.135	0.3268
Individual MHC diversity *density	1	2.009	0.1412	NA	NA	NA
F10 *density	NA	NA	NA	1	0.279	0.7573
Sex*density	1	0.140	0.1043	1	0.289	0.5927

^a corrected for F10; ^b corrected for sex. NA stands for not applicable variable within the model.

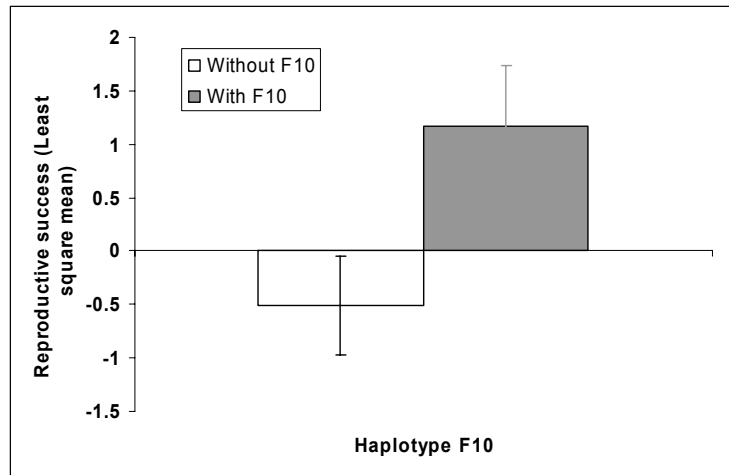
- Male mating behavior and breeding coloration

Performing the parenthood analysis for every typed egg, we assigned 9.8% of them to male sneaking behavior which is comparable to findings in natural populations (Rico et al. 1992; Largiadèr et al. 2001; Blais et al. 2004). The percentage of eggs fertilized in the nest of another male was negatively correlated with reproductive success (ANCOVA, $F_{1,46} = 5.558$, $p = 0.022$). Moreover, small fish achieved most of their fertilizations by sneaking behavior relative to bigger fish (ANCOVA, $F_{1,46} = 4.692$, $p = 0.035$). We also found that male density enhanced sneaking behavior (ANCOVA, $F_{1,46} = -2.587$, $p = 0.013$). Although not significant at 5%, males lacking the MHC haplotype F10 showed an increased tendency to gain fertilizations through sneaking (Welch t test, $t = 1.901$, $p = 0.065$).

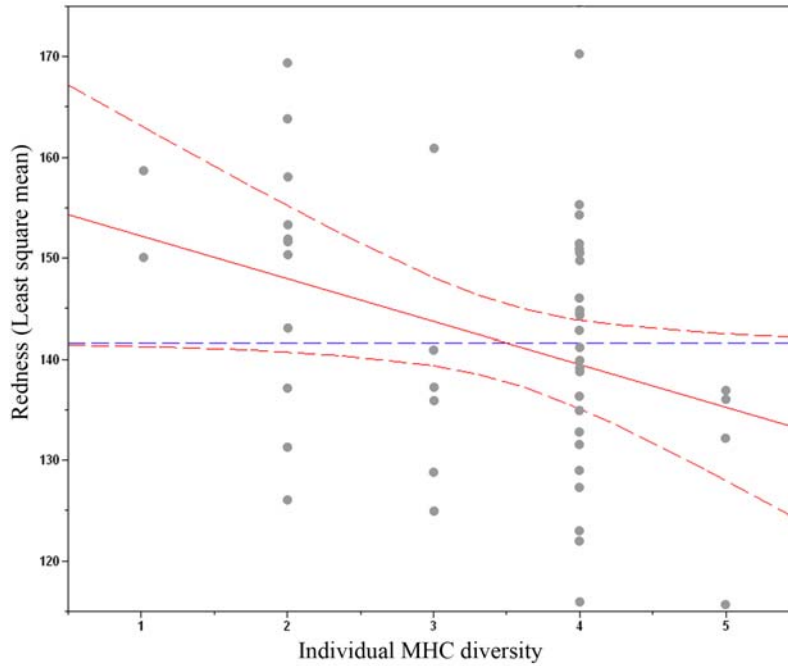
Finally, we investigated whether the male breeding coloration was related to MHC individual diversity, male density, parasite load, standard length and all double interaction with male density. Only MHC individual diversity was significantly negatively correlated to redness ($F_{1,46} = 4.353$, $p = 0.043$. Table 4. Fig. 6). Including F10 into the model did not reveal any significant effect (Table 4).



Chapter III Fig. 4: Relationship between the mean number of the parasite *Gyrodactylus* sp. and the occurrence of the haplotype F10 (standard error bars are depicted).



Chapter III Fig. 5: Individual reproductive success as least square means as a function of presence/absence of the MHC haplotype F10.



Chapter III Fig. 6: Male red coloration (least square means residuals) as a function of individual MHC class II B diversity.

Chapter III Table 3: Reproductive success of three-spined stickleback over one reproductive period. We re-conducted the analysis of covariance replacing MHC diversity with the variable “haplotype F10”. Since all females displayed the haplotype F10, a female specific analysis was not possible to test for the effect of F10. All the fish were first included then only males. NA stands for not applicable.

Variables	A) ALL FISH			B) MALES		
	d.f	F ratio	p-value	d.f	F ratio	p-value
Male density	1	9.425	0.0002	1	4.047	0.0504
Standard length ^{a,b}	1	24.259	<0.0001	1	23.267	<0.0001
Parasite load ^{a,b}	1	0.132	0.7170	1	3.719	0.0606
Haplotype F10	1	4.596	0.0353	1	6.013	0.0184
Sex	1	17.251	<0.0001	NA	NA	NA
Redness	NA	NA	NA	1	5.542	0.0233
Standard length ^{a,b} *density	1	0.012	0.9144	1	0.526	0.4723
Parasite load ^{a,b} *density	1	1.760	0.1794	1	0.951	0.3352
Haplotype F10*density	2	0.003	0.9532	2	0.091	0.7640
Sex*density	2	17.463	0.0001	NA	NA	NA
Redness * density	NA	NA	NA	1	2.425	0.1271

^a corrected for F10; ^b corrected for sex

Chapter III Table 4: Results obtained from an analysis of covariance on red intensity of the breeding coloration a) with individual MHC diversity and b) with F10 as variable.

a) Variables	ALL MALES/ REDNESS			ALL MALES/ REDNESS		
	d.f	F ratio	p-value	d.f	F ratio	p-value
Male density	1	0.033	0.8572	1	0.068	0.7955
Standard length ^{a,b}	1	0.053	0.8191	1	0.332	0.5672
Parasite load ^{a,b,c}	1	0.650	0.4242	1	2.305	0.1358
Individual MHC diversity	1	4.353	0.0425	NA	NA	NA
F10	NA	NA	NA	1	0.120	
Standard length ^{a,b} *density	1	1.100	0.3593	1	1.095	0.3613
Parasite load ^{a,b,c} *density	1	0.247	0.8631	1	0.760	0.5527
Individual MHC diversity *density	1	0.058	0.9436	NA		
F10 *density	NA	NA	NA	1	0.346	0.7925

^a corrected for F10; ^b corrected for sex; ^c corrected for individual MHC diversity; NA= not applicable.

Discussion

The idea that mate choice might be driven by genetic compatibility and/or good genes is generally accepted (Grob et al. 1998; Penn & Potts 1999; Landry et al. 2001; Mays & Hill 2004; Neff & Pitcher 2005; Byers & Waits 2006; Milinski 2006) but evidence remains unclear (von Schantz et al. 1996; Strohbach et al. 1998; Westneat & Birkhead 1998; Colegrave et al. 2002; Kokko et al. 2003; Welch 2003).

Previous studies often have not focused on fitness related genes and/or mate choice translated into Darwinian fitness. Our results represent the first evidence for a MHC haplotype, conferring resistance against a virulent parasite, that is directly translated into fish size and ultimately into Darwinian fitness.

We investigated, under semi-natural conditions, female stickleback mate choice based on MHC genes in relation to individual reproductive success, motivated by a previous experiment which used lab-bred fish (Kalbe et al. submitted). Here, the fish were wild-caught and exposed to their natural sympatric parasites. Using 9 microsatellites we were able to determine both parents for almost 4000 stickleback eggs. We found that, under natural conditions, female mate choice was not random with regard to MHC. Females significantly chose males that were neither MHC similar nor MHC dissimilar. We therefore conclude that females preferred mates with which they shared an intermediate individual MHC diversity. This result is consistent with previous flow channel experiments where females sticklebacks spent more time on the side of water from males offering a complementary set of MHC variants to potentially achieve an

intermediate individual MHC diversity in the offspring (Reusch et al. 2001a; Aeschlimann et al. 2003; Milinski et al. 2005). This preference was a significant prediction for the female's decision in which male's nest to spawn (Milinski et al. 2005). Here, we show that when females are confronted with a mating decision under natural conditions, with different males available, they follow our prediction for optimal immunogenetic make up for their offspring.

Various studies have suggested female mate choice for an intermediate individual MHC diversity (Jacob et al. 2002; Olsson et al. 2003; Bonneaud et al. 2006a; Forsberg et al. 2007; Schwensow et al. 2008). Such mate choice strategy is relevant when individuals with an intermediate MHC diversity are optimal with regard to parasite resistance, individual body condition, reproductive success and survival. Recently, there has been increasing evidence revealing the advantages for individuals with such MHC diversity (Reusch et al. 2001a; Aeschlimann et al. 2003; Wegner et al. 2003a; Wegner et al. 2003b; Bonneaud et al. 2005; Milinski et al. 2005; Madsen & Ujvari 2006; Forsberg et al. 2007; Kalbe et al. submitted; Wegner et al. 2008).

In addition, mate choice for good genes could arise if a specific MHC variant or haplotype confers a strong benefit against a common and virulent pathogen. Accordingly, our results showed that the probability of a male to be chosen by females was dependent on whether or not they bared a specific MHC haplotype. Consequently, males with this haplotype achieved a higher reproductive success. We reveal here that this specific MHC haplotype was conferring resistance against one of the most common parasistes- the monogenean *Gyrodactylus* sp. All the surviving females carried this specific haplotype. Females displayed a higher parasite load than males. Generally, this could reflect the need of high nutrient input for egg production achieved through intense feeding (Kalbe et al. submitted). Foraging behavior may drive exposure to an increased range of parasites, both trophically, and actively-transmitted and lead to selection against weaker females. We have not determined these parasites to species level. However, a previous survey revealed that the majority of *Gyrodactylus* sp. found in sticklebacks from lakes in our area are *G. gasterostei*, and it is know that *Gyrodactylus* species are relatively species-specific compared to other parasites (Raeymaekers et al.; Raeymaekers et al. 2006). Therefore, this parasite is one of the few stickleback-specific parasites of our system for which a host-parasite co-evolution based on genotype-genotype interactions can be expected. The three-spined stickleback is the final host of this parasite on which it feeds and reproduces. Several studies have reported the impact of *Gyrodactylus* species on host immune system activation (Buchmann & Lindenstrøm 2002; Lindenstrom et al. 2004; Collins et al. 2007). *Gyrodactylus* infection has been correlated with carotenoid input- the source of various male secondary sexual traits, like e.g. coloration (Kolluru et al. 2006). Such correlation between a specific MHC haplotype and a virulent parasite is a pre-requisite to negative-

frequency-dependent selection: one of the mechanisms invoked to explain MHC polymorphism.

Standard length was a strong determinant of reproductive success. This result supports the idea of adaptation to environmental selection regimes in sticklebacks (McPhail 1969; McKinnon et al. 2004). The size of an individual stickleback correlated with the possession of a specific MHC haplotype. Thus, we predict the following scenario for mate choice in stickleback; based on olfactory cues females approach males' nests, and based on visual cues inspect their condition (size, redness) before deciding whether or not to invest in reproduction with these males. Consistent with our prediction, where parasites affect the ability of individuals to reproduce, females should look for males carrying a specific resistant MHC haplotype.

Mainly two propositions have been put forward to explain why females should base their mating decision on MHC genes: to avoid inbreeding and/or to complement a compatible set of alleles (reviewed in Milinski 2006; Kempenaers 2007). Here, we can eliminate the possibility that our observations resulted from inbreeding avoidance since females mated randomly with regard to genetic background, potentially reflecting the lack of inbreeding risks at neutral loci. Interestingly, results suggest both, the compatible genes hypothesis as seen in the intermediate number of shared alleles between mates, and the good genes hypothesis as seen in the advantages of the males that carried the haplotypes F10. In fact, these two hypotheses might not be mutually exclusive (Colegrave et al. 2002). With this strategy, females achieve a two-fold immunogenetic advantages: resistance against a wide range of parasites while keeping the T-Cell repertoire depletion to a minimum level (Nowak et al. 1992; Woelfing et al. 2009) and against a currently common and costly parasite.

Such mating strategy is relevant since both additive effects and non additive effects of MHC genes are not mutually exclusive and here both provided increased resistance to parasites. Conceivably, as the prevalence of various parasites and pathogens changes in the environment, so will the survivorship value of the associated MHC variant. This could lead to negative-frequency-dependent selection and the fluctuation of allele frequencies through evolutionary time - mechanism thought to maintain MHC polymorphism. However, female mating strategy is not the only source of maintenance of MHC polymorphism.

As expected, male density increased female choosiness and increased the proportion of sneaking events. Sneaking behavior enabled lower quality males to circumvent mate choice and transmit genes to the next the generation maintaining MHC polymorphism by creating sexual conflicts.

Male density affected as well positively female reproductive success. During, their paternal care, males are less receptive to females (Wootton 1976). Increasing male density increased the likelihood that at least one male was receptive when females were ripe and ready to

spawn. This was reflected by 25% more nests present in high density enclosures compared to low density enclosure (76 vs. 57 nests detected within 4 weeks).

Males with a higher MHC diversity also displayed a duller breeding coloration and redness was associated with a lower reproductive success. We have previously demonstrated that red breeding coloration, which is estimated at the end of the experiment, lacks a timing component (Kalbe et al. submitted). We show here that when we estimated male's breeding coloration, the redder males were the ones of low quality: those males with low reproductive success, high parasitization and low MHC diversity. We proposed that these males followed a final investment strategy. To test our assumption, we compared the proportion of egg fertilized during the last week of the experiment over the entire reproductive period between the redder and the duller males. Redder males obtained 23% of their total reproductive success during the last week of the experimental period, whilst duller males obtained only 14%. Although it is an honest signal, the red breeding coloration might lead to misinterpretations of results if considered representative of a constant state of the fish.

As perspective for further studies, parasite/host MHC interaction, which directly translates into Darwinian fitness, could have implications on speciation. Females favored males with which they shared an intermediate MHC diversity. Such strategy avoids disrupting local adaptations of co-adapted gene complex (Hendry et al. 2000) and producing offspring of lower quality. Often assortative mating is thought to lead to purifying selection (Kirkpatrick & Nuismer 2004). However, this might not apply to MHC genes since allele frequencies vary rapidly between years responding to fluctuating parasite pressures as negative-frequency-dependent selection would envisage it (e.g. Westerdahl et al. 2004; e.g. Charbonnel & Pemberton 2005).

Thus, in this semi-natural field experiment, fish were exposed to the natural spectrum of parasites in their natal lake. We showed that female sticklebacks used an MHC-based mating strategy to transmit good genes. Using MHC signaling they can look for males in good condition providing the best paternal care and providing their offspring with a higher relative immunity in their pathogenic environment. Interestingly, assortative mating for MHC-adapted partners could influence our thinking in terms of understanding the rapid evolution of species.

Acknowledgments

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Chapter IV

Speciation accelerated and stabilized by pleiotropic MHC immunogenes.

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Abstract

Speciation and the maintenance of recently diverged species has been subject of intense research in evolutionary biology for decades. Although the concept of ecological speciation has been accepted, its mechanisms and genetic bases are still under investigation. Here we present a mechanism for speciation that is orchestrated and strengthened by parasite communities acting on polymorphic genes of the immune system. In vertebrates these genes have a pleiotropic role with regard to parasite resistance and mate choice. In contrasting niches, parasite communities differ and thus the pools of alleles of the adapted major histocompatibility complex (MHC) also differ between niches. Mate choice for the best-adapted MHC genotype will favor local adaptations and will accelerate separation of both populations: thus immune genes act as pleiotropic speciation genes – ‘magic traits’. This mechanism should operate not only in sympatric populations but also under allopatry or parapatry. Each individual has a small subset of the many MHC alleles present in the population. If all individuals could have all MHC alleles from the pool, MHC based adaptation is neither necessary nor possible. However, the typically small optimal individual number of MHC loci thus enables MHC-based speciation. Furthermore, we propose a new mechanism selecting against species hybrids. Hybrids are expected to have super-optimal individual MHC diversity and should therefore suffer more from parasites in all habitats.

Introduction

Almost half a century ago, Ernst Mayr (1963) predicted that the question of sympatric speciation would arise again: "One would think that it should no longer be necessary to devote much time to this topic, but past experience permits one to predict that the issue will be raised again at regular intervals." However, it is unlikely that he anticipated the collapse of the dogma of allopatric speciation he had advocated. Maynard-Smith (1966) affirmed Mayr's arguments and pinpointed the major objection against sympatric speciation: *i.e.* that it was not supported by any mechanism consistent with the known facts of genetics. This criticism should be reconsidered in light of the advances in genetics. We propose an explanation how speciation with, and even without geographical barriers, could arise, orchestrated and strengthened by the response of the highly polymorphic adaptive immune system of vertebrates to selection by parasites.

Various theories have been proposed to explain how species form (Ridley 2003). Only recently, the idea that ecology can play a major role in species formation has re-emerged (Schluter & Rambaut 1996; Funk 1998; Dieckmann & Doebeli 1999; Rundle et al. 2000; Dieckmann et al. 2004; McKinnon et al. 2004; reviewed in Rundle & Nosil 2005; and Bolnick & Fitzpatrick 2007). Ecological speciation is defined as a process by which barriers to gene flow evolve between sympatric populations due to adaptation to biotic or abiotic local environments. It results from divergent selection, even in the absence of geographical barriers (Mayr 1963; Schluter 2000; Schluter 2001; Rundle & Nosil 2005; Bolnick & Fitzpatrick 2007). Thus, according to ecological speciation theory, reproductive isolation takes its origin in divergent adaptation to contrasting environments. There are several excellent discussions of the main hypotheses explaining the speciation process (Schluter 2000; Dieckmann et al. 2004; Wu & Ting 2004; Rundle & Nosil 2005; Bolnick & Fitzpatrick 2007; Ritchie 2007). Several mechanisms have been proposed to explain divergence resulting from sexual selection such as the Fisher runaway process (Lande 1981) or sexual conflict (Parker & Partridge 1998; Gavrillets 2000; reviewed in Chapman et al. 2003). The major difference between these speciation models and the ecological speciation model is that divergence arises from interactions between sexes and is not linked with an environmental trait.

Typically, ecological speciation has been examined on the basis of morphological traits associated with adaptations to contrasting environments- e.g. beaks of Darwin's finches (e.g. Grant & Grant 1982; Schluter et al. 1985; e.g. Podos 2001), body size of scincid lizards (Richmond & Reeder 2002) or color and body armor in sticklebacks (McPhail 1969; Rundle et al. 2000; Schluter et al. 2004; Boughman et al. 2005). Ecologically-based disruptive selection requires genetic variation in a population. It can enhance this polymorphism by favoring

extreme phenotypes and thus genotypes. This is a prerequisite for eventual speciation. If we consider male ornamentation for which adaptation to the environment has selected for extreme features, e.g. conspicuous vs. cryptic, it is obvious that the two phenotypes are mutually exclusive, as a single individual cannot display both phenotypes at the same time. However, if two alleles have additive effects on the phenotype, disruptive selection would render the homozygotes fitter than the heterozygotes. Then one homozygote genotype would be fitter in one habitat, and the other in the second environment. This example suggests that to understand speciation without geographical barriers we should search for divergent phenotypes controlled by polymorphic genes. The candidate genes should be under positive selection and must respond to environmental variations on a short time scale.

According to Wu and Ting (Wu & Ting 2004), genes under positive selection are often non-essential in speciation because they are loosely coupled to reproductive isolation. However, pleiotropic effects of color pattern mimicry have been shown to influence mate choice in butterfly species (*Heliconius melpomene* and *H. cydno*) where reproductive isolation reinforces differentiation (Jiggins et al. 2001). Nonetheless, speciation genes are difficult to pinpoint. Various authors have questioned the potential existence of ecologically relevant traits simultaneously leading to assortative mating (e.g. Felsenstein 1981; Gavrillets 2005). Because of their double role, such traits are called “magic traits” (Gavrillets 2004). A single-gene speciation mechanism has been reported in snail lineages (Ueshima & Asami 2003). In these snails, a single gene controls shell chirality, an indispensable aspect of snail reproduction, because mating is prevented between individuals of different “handedness” by genital mismatch. Although the determinism of snail chirality is known, the respective selective pressure remains under debate, but predation could play this role (Dietl & Hendricks 2006).

Although predatory interactions can be invoked in the process of speciation (for instance Jiggins et al. 2001; Nosil 2004), host exploitation by parasites is more likely to drive divergence (Buckling & Rainey 2002). This idea takes its roots mainly in Haldane’s (1992) and Hamilton and Zuk’s (1982) work. Evolving parasites exert selective pressures likely to decrease Darwinian fitness not only directly, via reducing body condition for instance, but also indirectly by reducing selectivity and attractiveness in mate choice. Furthermore, resistance genes might have important effects on fitness and always remain heritable (Hamilton & Zuk 1982). The infection by parasites can be a strong selective pressure on the host's immune system. The interaction between host and parasite leads to continuous co-evolution. Since Hamilton and Zuk proposed their theory, empirical evidence has begun to accumulate (e.g. Milinski & Bakker 1990a; Moller 1990b; Zuk et al. 1990; Folstad & Karter 1992). It is not our aim to review the extensive literature dealing with speciation, because excellent work has already been done

(Schluter 2000; Schluter 2001; Wu & Ting 2004; Rundle & Nosil 2005; Bolnick & Fitzpatrick 2007; Ritchie 2007). Instead, we present a putative “magic trait”: the major histocompatibility complex (MHC), a complex of genes responsible for both adaptation to the environment and assortative mating.

MHC pleiotropy

One component of the environment that is a dynamic and tangible source of selection are parasites. The majority of organisms have developed genes involved in defense against pathogens: plants (Allen et al. 2004), invertebrates (Hoffmann et al. 1999) and vertebrates. For the latter, the polymorphic major histocompatibility complex (MHC) is a key component of the adaptive immune system (Janeway et al. 2001). Due to their function in self/non-self discrimination and their extreme polymorphism, MHC genes are ideal candidates to investigate ecological speciation. MHC molecules present pathogen-derived peptides to T-cells that subsequently initiate a specific immune response. There are two classes of MHC molecules, class I and II. While MHC class I molecules bind peptides derived from intracellular pathogens, e.g. viruses, MHC class II molecules present peptides from extracellular pathogens, e.g. macroparasites. Several studies have suggested that in different populations, the pools of MHC alleles are shaped by habitat specific parasites (Wegner et al. 2003b; Blais et al. 2007; Ekblom et al. 2007; Alcaide et al. 2008), as revealed by tight associations between MHC resistance alleles and parasites (Hill et al. 1991; Langefors et al. 2001; Bonneaud et al. 2005; Harf & Sommer 2005; Martin & Carrington 2005; Westerdahl et al. 2005; Milinski 2006; Loiseau et al. 2008). MHC genes have also been demonstrated to play a crucial role in mate choice in several species, e.g. birds (Bonneaud et al. 2006a), fish (Reusch et al. 2001a; Aeschlimann et al. 2003; Milinski et al. 2005; Forsberg et al. 2007), rodents (Yamazaki et al. 1976; Potts et al. 1991; Penn & Potts 1999; Penn 2002; Sommer 2005b) and primates (Schwensow et al. 2008). Maynard-Smith (1966) argued that a “gene pair adapting individuals to different niches may themselves cause assortative mating [...] seems very unlikely”. Here, however, we argue that the twofold role of MHC in mate choice and parasite resistance is an example for such pleiotropy.

Closer to the idea of ecological speciation, Summers and co-workers (2003) discussed the role of biotic conflicts between parasite virulence and host resistance genes in species divergence. When hosts are able to fight efficiently against local parasites, the authors predict that migrants and their hybrids are not able to resist parasitization sufficiently.

Can MHC genes fulfill Maynard Smith’s speciation paradigm?

Let us assume a simple model situation where an original population inhabits two contrasting but connected niches, e.g. sympatric lake populations. We focus on sympatric lake populations

although the mechanism is also valid and even facilitated under allopatric or parapatric speciation since we do not make any a priori assumption on the role of geographic barriers. Variation in ecological conditions (e.g. light levels, oxygen content, salinity or floristic assemblages) and community structure (e.g. competitors or predators), of the two habitats will influence the presence and abundance of certain intermediate hosts which are necessary for the maintenance of different parasite taxa and hence the probability of parasite transmission. Thus, the two contrasting habitats are likely to support distinct parasite communities; for example in sympatric whitefish and cichlids respectively (Thompson 1994; for examples see Knudsen et al. 2003; and Blais et al. 2007). Assuming that parasite-driven selection represents the major source of inter-individual MHC diversity, such contrast will lead to different pools and frequencies of MHC alleles in the adapted host populations. Via selection, each population maintains the alleles conferring higher fitness at a high frequency, i.e. the least parasite burden or reduced virulence effects on the host. When compared to the entire allelic population, intra-individual MHC diversity is limited to a small subset of alleles. From humans to salmonid fishes, the limited individual number of loci cannot display the entire potential MHC allele pool (Lawlor et al. 1990; Janeway et al. 2001; Robinson et al. 2003). We will discuss later why individuals have only a small subset of the allele pool. Only because of this restriction, the MHC can become locally adapted and a “magic trait”. Thus, local parasite-driven selection favours some alleles and disfavors others, and eventually confers the two host populations with different allele pools allowing the formation of distinct species. A “shared gene pool gives the species its identity” (Ridley 2003). If the two habitats would differ only in their parasite fauna, we would expect that the two new host species differ only in their MHC genetics. Being undistinguishable otherwise, they would be “cryptic species” (Mayr 1997). Many such cryptic but yet undetected species may exist.

For example, Blais and co-workers (Blais et al. 2007) investigated the role of MHC class II on two cichlid species in Lake Malawi. Cichlids provide an excellent study system for answering speciation related questions, because they are supposed to have diverged very recently (~15000 years ago). This study provides interesting results on selection acting on antigen binding sites of MHC genes and on parasite driving diversification at these immune genes. The authors did not restrict their investigation to MHC genes, but also found a correlation with parasite communities. In line with our predictions, they found that the parasite communities varied between the two species, which suggests that pathogen driven selection might have played a role in species differentiation. This result is the first hint that MHC genes can play a crucial role in species divergence. The two cichlid species are not “cryptic”, they differ also by the conspicuous colour of their dorsal fin, which is black or orange respectively (Blais et al. 2007).

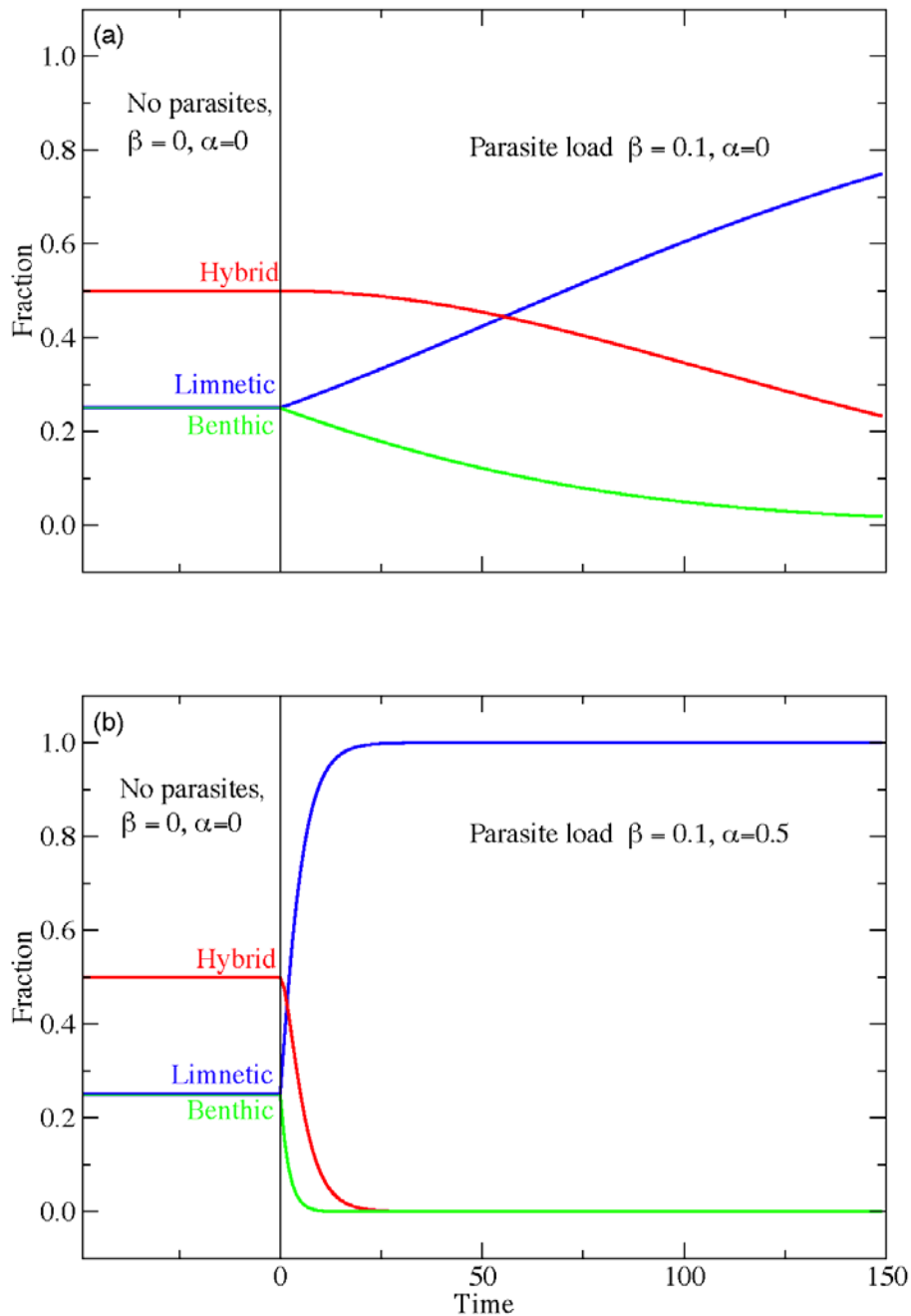
A model to illustrate the hypothesis

To illustrate the role of MHC pleiotropy on speciation dynamics, we assume three sympatric fish ecotypes, limnetic, benthic and hybrid inhabiting in a lake with two habitats, limnetic and benthic. The three types reproduce at a rate proportional to their Darwinian fitness r_l , r_b and r_h . Mating of two limnetic individuals produces limnetic offspring. If the limnetic type mates with the benthic type, hybrid offspring is produced. Mating of limnetic and hybrid produces 50% limnetic and 50% hybrid offspring. Mating of the benthic type is equivalent. The mating of two hybrids leads to 25% limnetic offspring, 25% benthic offspring and 50% hybrids.

Through its exploitation of host resources, and increase in predation probability, the local parasite burden decreases the fitness of the host type by different magnitudes and leads to selection of the most MHC adapted ecotype.

The evolutionary dynamics are equivalent in both habitats. Since both ecotypes have developed adaptive immune defences against their sympatric parasites, in the limnetic habitat an increased parasite load decreases the fitness of the benthic type the most and vice versa. Fitness is represented by $r_i = r^0 - s_i \cdot \beta$, where r^0 is the fitness in the absence of parasites, s_i is the parasite influence on type i , and β is the parasite load. Figure 1a shows that the selection exerted by a fixed parasite community on MHC genes leads to an increase of the most adapted MHC genotype (the limnetic) and a decrease of the least adapted (the benthic) in the limnetic habitat.

In the absence of selection ($r_l = r_b = r_h$), mating is random. However, if there is a better adapted MHC genotype, then non-random female MHC-based mate choice is possible. We assume that with probability $\alpha > 0$, the limnetic type is chosen as a mating partner by limnetic females. The reproductive dynamics can be captured by a set of differential equations for the fractions x of the limnetic, hybrid, and benthic types in the population (see box1). In the limnetic habitat, the limnetic ecotype is most adapted and the benthic type least. Therefore, limnetic females should favour limnetic males, i.e. males that would provide the best immunogenetics make up to the offspring. Figure 1 b illustrates the acceleration of the population differentiation under the pleiotropic action of MHC genes with regard to parasite resistance and mate choice.



Chapter IV Fig. 1: MHC based mate choice accelerates population separation. (a) without parasites and mate choice, selection is neutral. At time $t=0$, parasites are added ($\beta=0.1$). In the limnetic habitat, the better adapted limnetic MHC genotypes increase in frequency until they dominate the population. (b) with MHC based mate choice ($\alpha=0.5$), the dynamics are markedly faster for the same parasite load, and the final dominance of the limnetic type is obtained much earlier. If the same genes responding to environmental pressure (parasites) are involved in mate choice, it will accelerate the population separation based both on MHC genes and local adaptations (Parameters $r_0=1, s_L=0.5, s_H=0.75, s_B=1, a=0$, parameter order from (Scharsack et al. 2007)).

Can MHC genes meet different theoretical scenarios?

Assortative vs. disassortative mating. Our proposed scenario assumes assortative mating within morphs or species. As in classical host parasite co-evolution models, we focus on selection on the host because most parasites and pathogens have a shorter generation time than their exploited host (Frank 1996; Lively 1999). Recently, new insights from Nuismer *et al.*'s work (2008) shows that co-evolution between host and parasites could lead to assortative mating, in particular when the matching allele model and within group mating are considered. This new insight is consistent with previous studies on MHC revealing the tight association between specific alleles and specific parasites (Hill *et al.* 1991; Langefors *et al.* 2001; Bonneaud *et al.* 2005; Harf & Sommer 2005; Martin & Carrington 2005; Westerdahl *et al.* 2005; Milinski 2006; Loiseau *et al.* 2008). Theoretical work predicted a purifying effect of assortative mating (Kirkpatrick & Nuismer 2004). However, this might not apply to MHC genes since allele frequencies vary rapidly between years responding to fluctuating parasite pressures (e.g. Wegner 2004; Westerdahl *et al.* 2004).

Sexual selection and speciation. Several theoretical papers suggested that sexual selection alone is unlikely to drive speciation (for reviews see for instance Gavrillets 2003; Coyne & Orr 2004), whilst others argue that it is possible (Lande 1989; Turner & Burrows 1995; Chapman *et al.* 2003; Ritchie 2007). Once more, MHC genes meet the necessary condition to respond to both sexual and natural selection in the process of speciation. Indeed, MHC polymorphism driven by parasite selection is often explained by negative frequency dependent selection, which can lead to disruptive selection. Moreover, specific MHC alleles, considered under negative-frequency dependent selection, have been shown to correlate with important male secondary sexual traits such as the snood length in turkey (Buchholz *et al.* 2004), or the red throat of sticklebacks (Jäger *et al.* 2007).

A novel mechanism selecting against hybrids in vertebrates

To mount an efficient MHC based immune response it is important to carry not only the right quality of MHC alleles, i.e. those providing resistance against the local infectious diseases, but also the right number of different MHC alleles, i.e. the optimal individual MHC diversity (Nowak *et al.* 1992; De Boer & Perelson 1993; Woelfing *et al.* 2009). MHC molecules selectively present peptides of pathogens. To be able to resist a broad range of pathogens, one would expect selection to have favoured high individual MHC diversity, i.e. high individual MHC heterozygosity. To be able to recognize all possible infectious diseases, each individual should have all MHC alleles from the population pool. However, an increased number of MHC variants may not only bind more foreign but also a greater variety of self-derived peptides,

raising the risk of autoimmune responses. T-cell clones that recognise self-peptides bound to MHC molecules are thus eliminated from the initially highly variable repertoire, thereby reducing the T-cell clone repertoire that is finally available for parasite recognition (Lawlor et al. 1990; Janeway et al. 2001). A balance between the two opposing selective forces of either broader pathogen detection or increased T-cell clone elimination may lead to an optimal rather than a maximal individual MHC diversity as theory predicts (Nowak et al. 1992; De Boer & Perelson 1993; reviewed in Milinski 2006; Woelfing et al. 2009). In fact, individuals with an intermediate rather than a maximised number of different MHC alleles have been shown to be more resistant to infectious diseases (Wegner et al. 2003a; Wegner et al. 2003b; Bonneaud et al. 2004; Buchholz et al. 2004; Madsen & Ujvari 2006). Individuals with a sub-optimal number

Box1:

The fractions of the limnetic, hybrid and benthic ecotypes in the population, x_l, x_h and x_b , change due to selection for the most adapted ecotype. This dynamics is determined by the differences in the fitness r_i of the three ecotypes and the probability for assortative mating α . It can be modelled by the three differential equations

$$\begin{aligned}\dot{x}_l &= x_l \cdot r_l \left[\alpha + (1-\alpha) \left(x_l + \frac{x_h}{2} \right) \right] + x_h \cdot r_h \left[\frac{\alpha}{2} + (1-\alpha) \left(\frac{x_l}{2} + \frac{x_h}{4} \right) \right] - x_l \cdot \phi \\ \dot{x}_h &= x_l \cdot r_l \left[(1-\alpha) \left(\frac{x_h}{2} + x_b \right) \right] + x_h \cdot r_h \left[\frac{\alpha}{2} + (1-\alpha) \left(\frac{x_l}{2} + \frac{x_h}{2} + \frac{x_b}{2} \right) \right] + x_b \cdot r_b \left[\alpha + (1-\alpha) \left(x_l + \frac{x_h}{2} \right) \right] - x_h \cdot \phi \\ \dot{x}_b &= x_b \cdot r_b \left[(1-\alpha) \left(x_b + \frac{x_h}{2} \right) \right] + x_h \cdot r_h \left[(1-\alpha) \left(\frac{x_b}{2} + \frac{x_h}{4} \right) \right] - x_b \cdot \phi\end{aligned}$$

Here, $\phi = x_l \cdot r_l + x_h \cdot r_h + x_b \cdot r_b$ is the average fitness, which ensures that normalization is preserved, $x_l + x_h + x_b = 1$. For illustration, let us discuss the first term in the first equation. At rate $x_l \cdot r_l$, limnetic females reproduce. With probability α , a limnetic male is chosen as partner, leading to limnetic offspring. With probability $1-\alpha$, partner choice is random. In this case, limnetic offspring is produced if the male is limnetic or, with 50% probability, if the male is a hybrid. Thus, this term describes the increase of the limnetic type when the female is limnetic. The other terms follow equivalently.

Neutral selection ($r_l = r_h = r_b$) and random mating ($\alpha = 0$) lead to the usual Mendelian inheritance. For random mating ($\alpha = 0$) under selection, the differential equations model natural selection with random mating within one habitat: The type with the highest fitness will slowly spread in the population. When mating is assortative ($\alpha > 0$), the probability that more adapted individuals are involved in reproduction is increased and the speed of selection is thus significantly accelerated, cf. Dieckmann & Doebeli (1999).

of MHC alleles have more parasites, as they have too few different MHC molecules to present foreign peptides to their t-lymphocytes. Individuals with a super-optimal number of MHC alleles suffer from more infections as their T-cell repertoire has been reduced too much to resist the MHC recognised parasites. Hence, the typically small individual MHC diversity is an optimum and MHC based local adaptation and thus MHC based speciation is possible.

What would happen to hybrids between two new species which differ in their MHC allele pools to a large extent, as they are each adapted to their local parasite fauna? Even if mate choice aims to optimize MHC diversity in offspring (Aeschlimann et al. 2003; Milinski et al. 2005), this would be rarely achievable if two largely divergent sets of MHC alleles are combined from two mates of different species: a super-optimal individual MHC diversity in the hybrid offspring should be the rule. This means that hybrids would have enough different MHC molecules for parasite detection but are lacking T-cell clones to destroy their infected cells. These hybrids should suffer in all habitats from a higher parasite load than is found in each of their parents. There is suggestive evidence that agrees with this prediction, as the “wormy mice” in hybrid zones (Sage et al. 1986). A super-optimal individual MHC diversity should be a common disadvantage of species hybrids in vertebrates, resulting in elevated parasite loads. However, this prediction awaits empirical testing.

Conclusion

Adaptive immune genes could trigger and accelerate speciation processes via their pleiotropic role on parasite adaptation and assortative mating. This concept provides a mechanistic explanation for sympatric parasite-driven speciation. It can easily be extended to other types of speciation processes, reinforcing already pre-existing genetic divergence, e.g., in allopatry. MHC-based assortative mating will enhance the pace of population separation, resulting in speciation. Because of super-optimal individual MHC diversity of hybrids and an associated higher parasite load, there should be selection against hybridization. To sum up, MHC pleiotropic effects can induce and maintain reproductive isolation between populations, even in the absence of geographical boundaries.

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Chapter V

Ecological pleiotropy: pre- and post- zygotic reproductive isolation through MHC mediated selection.

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Abstract

The extreme polymorphism of the major histocompatibility complex (MHC) is probably maintained by parasite-mediated selection and sexual selection. These synergic forces could further promote the evolution of assortative mating in host populations as a mechanism through which genetically locally-adapted offspring can be produced. In a multidisciplinary approach, using three-spined stickleback populations, we investigated whether fish adaptation was reflected in female mate choices. Differences in morphology, information from stable isotope ratios and parasites communities indicated individual habitat adaptation. Presumably as a consequence of contrasting parasite communities, the two fish populations showed distinct MHC allele pools differing in diversity and constitution. To test for pre-zygotic reproductive isolation including MHC signaling, we used a flow channel mate choice experiment, based on olfactory cues. Females significantly chose fish from their home habitat more often, exhibiting a genetically-based reproductive isolation. We also propose that MHC-maladapted lake/river hybrids could be selected against by parasite pressure. Pre- and post-zygotic MHC-mediated selection could accelerate the pace of speciation and could contribute to the formation and maintenance of recently diverged species. The pleiotropic MHC genes could thus be regarded as ecologically relevant speciation genes and magic traits.

Introduction

Various theories have been proposed to explain how species form (Ridley 2003). Recently, the idea that ecology can play a major role in species formation has re-emerged (Schluter & Rambaut 1996; Rundle et al. 2000; Dieckmann et al. 2004; McKinnon et al. 2004; Bolnick & Fitzpatrick 2007). Ecological speciation is defined as the process by which reproductive isolation between populations may evolve from adaptation to different environments. A textbook example is represented by the Darwin's finches inhabiting the Galápagos Archipelago (Grant & Grant 1982; Grant 1986). Niche specialization resulting from competitive interactions for resources resulted different beak sizes and shapes evolving within the finches. Subsequent assortative mating for beak morphology then led to reproductive isolation, and finally to speciation (Podos 2001).

As with competitive interactions, the parasite community represents a component of the environment providing a tangible and dynamic source of selection (Hamilton & Zuk 1982; Peters & Lively 1999; Summers et al. 2003; Poulin 2006). Ecological conditions such as oxygen concentrations, floristic assemblages or community structure, e.g. competitors or predators, of macro- and micro-habitats are likely to influence the presence and abundance of certain intermediate hosts. These differences may therefore result in the development and maintenance of different parasite taxa (Thompson 1994).

Unlike invertebrates, jawed vertebrates have developed an adaptive defence system against parasites, the major histocompatibility complex (MHC) (Janeway et al. 2001). MHC molecules function as peptide shuttles that transport antigens from the cytoplasm and display them at the cell surface. T-cells bind pathogen-derived peptides and subsequently initiate a specific immune response. Particularly, MHC class II genes are involved in the immune answer against extra-cellular parasites by coding for MHC molecules that present exogenously derived peptides to CD 4+ T helper cells (Janeway et al. 2001). This gives MHC genes a key role in the onset and modulation of both antibody- and cell-mediated specific immune response.

MHC genes are the most polymorphic genes found in vertebrates: this high intra and inter-individual polymorphism is thought to be parasite-mediated and to be maintained by negative-frequency-dependent selection, overdominance and habitat heterogeneity (for review see Apanius et al. 1997; Milinski 2006; Piertney & Oliver 2006). Several studies have suggested that single MHC alleles or MHC haplotypes are associated with either susceptibility or resistance to disease- a pre-requisite for negative-frequency-dependent selection (e.g. Paterson et al. 1998; Langefors et al. 2001; Grimholt et al. 2003; e.g. Bonneaud et al. 2006b). Overdominance (Doherty & Zinkernagel 1975) suggests that heterozygous individuals are able to present a wider array of antigens to T cells and therefore to recognise more parasite species. Although overdominance has been both suggested and experimentally demonstrated

(e.g. Nevo & Beiles 1992; Carrington et al. 1999; Penn et al. 2002; e.g. McClelland et al. 2003; Froeschke & Sommer 2005; Hraber et al. 2007), it remains controversial. Indeed multiple infections have demonstrated both an advantage of intermediate MHC diversity in sticklebacks (Wegner et al. 2003a; Wegner et al. 2003b) and pythons (Madsen & Ujvari 2006) and decreased resistance in heterozygous mice (Ilmonen et al. 2007). It is worth noting that De Boer *et al.* (2004b) showed in a mathematical model, that overdominance alone does not explain high MHC polymorphism, suggesting that there are other selective mechanisms. Finally, various recent studies investigating MHC variation in neighboring populations revealed that habitat heterogeneity shapes MHC composition, probably due to contrasting parasites communities (Blais et al. 2007; Ekblom et al. 2007; Alcaide et al. 2008; Babik et al. 2008). These three mechanisms are not mutually exclusive. Thus, distinguishing between different and possibly interacting modes of selection is difficult.

Due to their: i) function in self/non self discrimination; ii) extreme polymorphism (under balancing selection); and iii) capacity to respond to environmental variations on a short time scale, MHC genes are ideal candidates to investigate ecologically-mediated genotypic adaptation in recently diverged species or in diverging populations and by then tracking the first steps of speciation.

Following the biological concept of species, populations which do not interbreed in sympatry can be considered as species. In this concept, reproductive isolation is a determinant factor. The three-spined stickleback, *Gasterosteus aculeatus*, serves as prime example for rapid genetic divergence. Throughout the northern hemisphere, it is thought to have colonized freshwater habitats following the last glaciations from adjacent estuarine or marine refuges (review in McPhail 1994). Over this short time period (ca.12 000 y), ancestral marine genotypes rapidly differentiated into several ecotypes, which differ remarkably in numerous morphological traits, behavior and ecology (Boughman 2001; Schluter et al. 2004; Leinonen et al. 2006; Taylor et al. 2006; Kitano et al. 2007; Marchinko & Schluter 2007) as a result of divergent selection (Schluter & Rambaut 1996; Vines & Schluter 2006).

Reusch *et al.* (2001b), investigated sixteen populations in Northern Germany, and revealed the dominant role of habitat in genetic divergence during the post glacial recolonization. Since there are no major obstacles (e.g. large dams or waterfalls) between lakes and rivers in this area, they regarded these populations as sympatric. This study, based on microsatellites, suggested that selection on organismal traits resulted in mating barriers and reduced gene flow. The genetic distance between ancestral marine populations and both lake and river populations was very similar, leading Reusch *et al.* to suggest the occurrence of a single colonization event. The most probable explanation for the divergence is therefore rapid

adaptive changes driven by niche specialization into lake and river ecotypes, reinforced by reproductive isolation.

There is growing evidence for MHC-based mate choice in mice (Yamazaki et al. 1976; Potts et al. 1991; Penn & Potts 1998), in birds (Richardson et al. 2005; Bonneaud et al. 2006a) in fish (Reusch et al. 2001a; Aeschlimann et al. 2003; Milinski et al. 2005; Forsberg et al. 2007; Consuegra & Garcia de Leaniz 2008), in non-human primates (Schwensow et al. 2008) and human (Wedekind et al. 1995; Jacob et al. 2002). Although these studies agree regarding the role of MHC genes in mate choice, the underlying ultimate aim can vary from inbreeding avoidance, to obtaining the best immunogenetic make up for the offspring (Potts et al. 1991; Aeschlimann et al. 2003; Wegner et al. 2003a; Milinski et al. 2005; Ilmonen et al. 2008).

Female choice has been intensively investigated in the three-spined stickleback (Milinski & Bakker 1990a; Milinski & Bakker 1992; Bakker 1993; Bakker & Mundwiler 1994; Boughman 2001; Aeschlimann et al. 2003; Kitano et al. 2007). In sticklebacks females assess their potential partners' MHC make-up via perception of corresponding peptide ligands in odor signals (Milinski et al. 2005). Olfactory recognition was shown to maintain reproductive isolation between sympatric benthic and limnetic sticklebacks from Paxton Lake (British Columbia, Canada) suggesting a role of MHC genes (Rafferty & Boughman 2006).

The fact that parasites can drive sexual selection in hosts (Hamilton & Zuk 1982; Milinski & Bakker 1990a; Moller 1990a) suggests that MHC-based mate choice promote the evolution of disassortative mating in host populations as a mechanism through which genetically resistant offspring can be produced. This possibility has been supported via simulation studies and suggested in some empirical systems where somewhat disassortative mating by disease resistance genotypes has been documented (Potts et al. 1994; Penn & Potts 1999; Reusch et al. 2001a; e.g. Aeschlimann et al. 2003; Howard & Lively 2004; review in Milinski 2006). Nevertheless, the generality of this phenomenon is unclear. For instance, what would happen to migrants or hybrids of two species if they are not able to sufficiently resist parasitization in a given habitat? In that case, assortative mating for immuno-resistance might be expected to avoid disruption of locally adapted genes. Therefore, the pleiotropic role of MHC genes in resistance to parasites and mate choice could reduce gene flow and thus enhance the process of speciation (Summers et al. 2003; Blais et al. 2007; Eizaguirre et al. 2008a). Models have shown when mate choice is based on ecologically important trait, divergence in that trait can accelerate allopatric speciation and facilitate sympatric speciation. However, identification of a "magic trait" (Gavrilets 2004) on which assortative mating and adaptation are based have seldom been reported (Jiggins et al. 2001; Podos 2001; Vines & Schluter 2006) and often the genetic basis not known.

Here, we investigated stickleback populations from a connected lake/river system, which are exposed to different parasites communities (Kalbe et al. 2002). This differential exposure could result from niche utilization. We used an integrative approach to investigate whether MHC genes could co-evolve with diet specialization or niche utilization using stable isotopes and morphological characteristics. Then, we compared parasite communities and tested whether MHC could reflect local adaptation to these two different but connected habitats. A previous study has shown fish from lake to have a higher individual MHC diversity (Wegner et al. 2003b). However, the allelic pools, reflecting the potential local adaptation, had not been investigated. Finally, to conclude that host immune system/parasite interaction could lead to the divergence of populations, we performed a mate choice experiment based on olfactory cues. In other words, we tested whether the twofold role of MHC (parasite resistance and mate choice) represents an example of pleiotropic genes adapting individuals to different niches, and then subsequently causing assortative mating potentially driving the system to speciation (Maynard Smith 1966; Summers et al. 2003; Eizaguirre et al. 2008a). In other words, we aim at demonstrating that MHC genes could be regarded as “magic traits” and could be at least as relevant trait as morphological characteristics.

Material & Methods

Fish sampling and dissections. We sampled three-spined sticklebacks (*Gasterosteus aculeatus*) from two habitats, a small, slow-flowing river (Malenter Au, 54°12'16.19"N, 10°33'32.93"E, Germany) and a lake (Großer Plöner See, 54° 9'21.16"N, 10°25'50.14"E, Germany) from an already described drainage system (Reusch et al. 2001b; Kalbe et al. 2002; Wegner et al. 2003b; Rauch et al. 2006b). Fish were sampled during winter 2007 (January and March) in order to assure that fish were from the same age-cohort. Fish were killed by an overdose of MS222. All external, internal macroparasites and ciliates were determined to the lowest taxonomic level possible using dissection microscopes (Kalbe et al. 2002). Fish mass (\pm 0.1 mg), standard length (\pm 1 mm) and lateral plate number were recorded.

Stable isotopes. We used stable isotope analysis (SIA) to examine whether sticklebacks from lake and river habitats showed clear differentiation in diet and habitat use. Previous studies have used SIA to reveal individual migration between ecologically-contrasting but connected habitats in aquatic consumers species as diverse as European eels (Harrod et al. 2005) and Galápagos sea lions (Wolf et al. 2008). A section of caudal muscle was dissected, and oven dried for 24 hours at 65 °C. Samples were ground, weighed into tin cups and analyzed for carbon ($\delta^{13}\text{C}$) and nitrogen ($\delta^{15}\text{N}$) stable isotope ratios and elemental C and N composition (see Harrod et al. 2005 for details). Repeated analyses of internal standards showed precision

of < 0.1 ‰ (carbon) and < 0.3 ‰ (nitrogen). $\delta^{13}\text{C}$ data were arithmetically lipid-normalized following Kiljunen *et al.* (2006).

MHC diversity and microsatellites typing. DNA extractions from tail fin tissue were performed using the Invisorb DNA Tissue HTS 96 Kit / R (Invitex, Germany) on an automated platform (Tecan, Switzerland) following the manufacturer's protocol. All fish were typed for 9 microsatellites combined in 2 different PCR multiplex protocols (Kalbe *et al.* submitted). We used these markers to calculate an individual heterozygosity index (Coulson *et al.* 1998) and for population structure analysis. The MHC class IIB diversity was determined by reference strand conformation analysis (RSCA) according to Lenz *et al.* (in prep). We amplified the exon 2 of MHC class IIB genes that encodes for the peptide-binding groove. This exon has previously been suggested, in this species, to be under parasite mediated positive selection as revealed by a high ratio of nonsynonymous over synonymous substitutions (d_N/d_S) (Reusch *et al.* 2004) .

Odor based mate choice test. For mate choice trials, further fish collections took place between June and July 2006 and 2007. After capture, fish were held singly in 16 l tanks in summer conditions (day light cycle 16:8h, 18°C air and water temperature), and males were provided with artificial nesting material (sand and nylon threads). All experimental tests were conducted within 10 days to 6 weeks post-collection. The fish were fed twice daily with live food consisting of chironomid larvae, tubifex, daphnids or artemia.

For the mate-choice tests, a randomly-picked gravid female was placed in the choice chamber of a flow channel. The chamber had two inlets through which a continuous passage of neutral water was generated (for details see Reusch *et al.* 2001a; Aeschlimann *et al.* 2003; Milinski *et al.* 2005). After an acclimatization period of 5 min, male water was added via the inlets: on one side, water originating from a male of the same habitat as the test female, and on the other male water from the other habitat. As nests appeared to be the focus of MHC and male signaling factor (Milinski *et al.* 2005; Sommerfeld *et al.* 2008), the water used for choice tests was taken from directly above a male's nest and only from males that glued their nest on the day of the test. The time the test female spent in the quarter close to the respective inlet was measured over a 5 min period. After a 2 min break with neutral water, the sides of the two different water sources (home habitat, foreign habitat) was exchanged and another 5 min period was measured to account for any side-preferences of the females. All tests were conducted blind with respect to the side water sources and were recorded with a digital processing video camera (Panasonic GP-KR222, Secaucus, NJ, USA).

For these tests a pair of males was always tested with a female from the lake habitat and the same pair with a female the river habitat. With this procedure we obtained 9 quadruplets (i.e.

36 fish, 18 tests). Moreover, due to a limited pool of experimental animals, in two cases this was not possible: one pair of males could only be tested with a lake female and another pair of males was only tested with a river female, this resulted in a total of 20 trials. Tests were regarded as valid only if the females spawned within 24 hours after the trial, which verifies the relevance of the females' choice (Reusch et al. 2001a; Aeschlimann et al. 2003; Milinski et al. 2005; Sommerfeld et al. 2008). Choice in the flow channel has been verified as a prediction of ultimate choice of which male to spawn with (Milinski et al. 2005).

Statistical analysis. Except if stipulated, statistical analyses were conducted using R statistical package (v. 2.5.0) for Windows XP and Primer v6 (Clarke & Gorley 2006). Normality of distributions and variance homoscedasticity were verified and tests conducted accordingly.

- **Morphology and stable isotopes**

We performed a discriminant analyses with weight, standard length and number of lateral plates as independent variables to examine whether these characteristics would be sufficient to identify the origin of fish collected from the wild. Since sample size differed between habitat and because of variance heterogeneity of lateral plates, we used a quadratic discriminant function. We then performed a similar analysis using $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ and the elemental ratio of carbon and nitrogen (C:N) recorded from each individual as predictors. We also estimated individual trophic position following Post (2002), using mean $\delta^{15}\text{N}$ values for mollusks collected from the same habitats as sticklebacks.

- **Population structure**

Species can be defined as populations sharing a common pool of genes (Ridley 2003). Therefore population structure is an essential feature to be investigated. Deviation from Hardy-Weinberg equilibrium was tested using the Markov Chain permutation test implemented in GENEPOP (Raymond & Rousset 1995). Reusch *et al.* (2001a) suggested that estimators assuming an infinite alleles model such as F_{ST} may perform better than alternative measures considering the stepwise mutation model in recently diverged populations e.g. R_{ST} (Slatkin 1995). Therefore, we quantified population differentiation using standardized allelic variance F_{ST} (Weir & Cockerham 1984) using GENETIX software v. 4.05.02 (Belkhir et al. 1996-2004). Wright's F-statistics were estimated between the 2 study sites and the two sampling periods. To confirm population clustering we used the software STRUCTURE 2.2 (Pritchard et al. 2000) and the admixture model of the software. We repeated each run 6 times with 10000 equilibration steps followed by a run length of 10000 Markov Chain Monte Carlo simulations. To determine the number of populations (K) most likely to explain the genetic data, we used

the posterior probabilities $\ln P(x|k)$. This also permits detection of potential hybridization between the two fish populations.

- **MHC pools & parasite data**

In order to estimate population-divergence in MHC class II B genes, we used an analysis of similarity (ANOSIM) based on a Bray-Curtis similarity matrix. MHC alleles were coded as binomial (presence/absence) variables. We also compared individual MHC diversity between ecomorphs.

Difference in parasite communities between habitats was tested with a further ANOSIM using parasite species as variables. In order to quantify the total parasite load of each fish, the Shannon index, which accounts for both abundance and evenness of the species present, was calculated following a fourth root transformation of parasite abundance. This allowed us to summarize different combinations of parasites for each stickleback and to make a quantitative comparison of their total parasite burden.

- **Olfactory-based mate choice**

We use a two-step approach to analyze female mating decision. First, with a binomial test, we investigated whether females more often chose males from the same habitat. Secondly, since the time spent in front quarters of the choice chambers was normally distributed we used t tests to compare female preference.

Results

Fish morphology and stable isotopes

- **Morphology and Lateral plates**

To test whether fish presented different physical characteristics, we regressed the weight of the fish over their standard length. This represents a diagnostic tool to estimate morphological differences. We tested whether the shape was correlated with the sex, the habitat of origin, the parasite load and the individual MHC diversity (simple and quadratic term). We found that only the variable habitat was significant: fish from the river were smaller and more robust than fish from the lake (ANCOVA, $F_{1,129}=15.09$, $p=0.0002$).

We tested whether the number of lateral plates was function of the habitat of origin, sex and their interactions. Results indicated that habitat was the only important variable, and lake sticklebacks had a higher number of lateral plates than fish from the river (ANCOVA, $F_{1,129}=31.96$, $p<0.0001$). Indeed, river fish form a monomorphic population where only low plated fish exist, whereas lake fish exhibit a broader distribution of plate number from low plated to high plated.

	HABITAT OF CAPTURE	LAKE	RIVER	%CORRECT
<i>Morphology</i>	Lake	56 (56)	6 (6)	90 (90)
	River	11 (9)	56 (58)	84 (86)
	Total	67 (65)	62 (64)	87 (88)
<i>Stable isotopes & C:N</i>	Lake	58 (58)	2 (2)	98 (98)
	River	0 (0)	68(66)	100 (100)
	Total	58 (58)	69 (66)	100 (100)

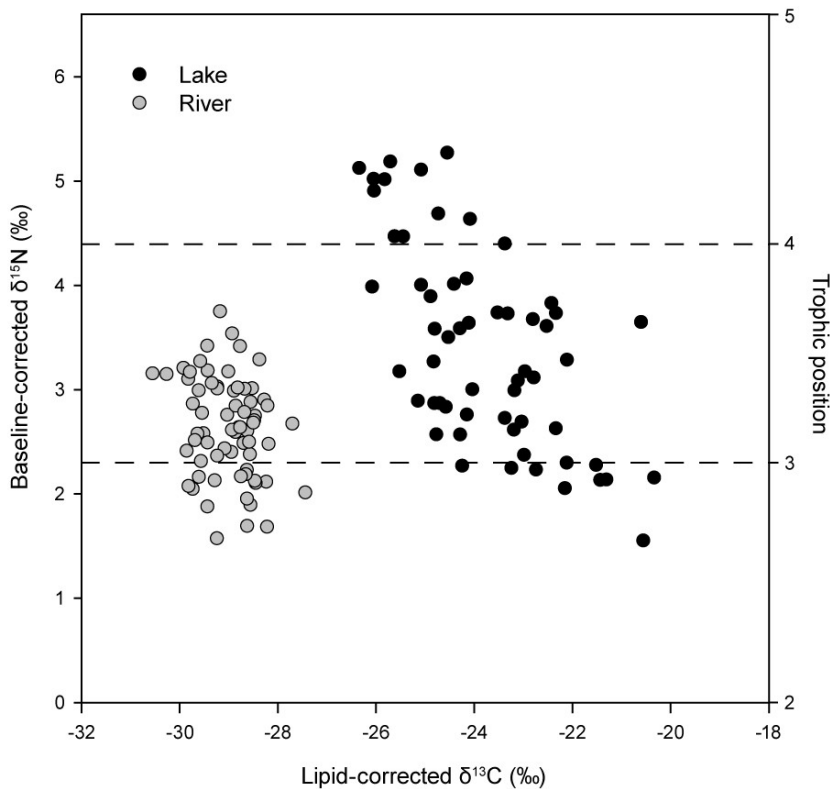
Chapter V Table 1: Table summarizing classification success and jackknifed classification success (in brackets) of the discriminant function analysis using morphological measurements and stable isotope signatures as the predictor variable. Classification success describes the predictive accuracy with which an individual is correctly associated with any of the two habitat of interest that were defined *a priori*.

The quadratic discriminant analysis performed on morphological characters showed a significant difference between fish from both habitats (Wilk's $\lambda = 0.687$, $F_{3,129} = 19.6$, $p < 0.0001$). The overall jackknifed classification was 87% successful in assigning fish to their original habitat (Table 1).

- $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ isotopic signatures

Comparison of $\delta^{13}\text{C}$ (lipid-corrected) and $\delta^{15}\text{N}$ (baseline-corrected) showed that lake and river sticklebacks inhabited completely contrasting isotopic space (Figure 1). Fishes from each habitat showed distinct $\delta^{13}\text{C}$ values with no overlap between habitats, allowing clear differentiation between habitats (Non parametric Mann-Whitney test, $Z = 3944$, $p < 0.0001$). Baseline corrected $\delta^{15}\text{N}$ values, reflecting individual trophic level also displayed significant differences between both habitats (Student test, $t = 5.51$, $p < 0.0001$). Interestingly, fish from the lake showed larger variance than fish from the river in $\delta^{15}\text{N}$ showing a higher degree of individual trophic variation in lake sticklebacks ($\sigma^2_{\text{lake}} = 0.919$; $\sigma^2_{\text{River}} = 0.231$; Levene's test $F_{1,129} = 29.72$, $p < 0.0001$. Figure 1). Calculation of individual trophic position (TP) showed that TP ranged by 1.9 trophic levels in lake fish, compared to 0.99 trophic levels in river sticklebacks. A quadratic discriminant analysis using lipid-corrected $\delta^{13}\text{C}$ and non-corrected $\delta^{15}\text{N}$ as well as C:N ratios (a correlate of lipid concentration) highlighted even stronger differences between ecotypes than the morphology did (Wilk's $\lambda = 0.119$, $F_{3,120} = 294.8$,

$p < 0.0001$). The jackknifed classification correctly affiliated 100% of the fish to their home habitat (Table 1).



Chapter V Fig. 1: Variation in $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ in lake and river habitats. $\delta^{15}\text{N}$ values were corrected for variation at the base of the food chain by subtracting the mean value for long-lived primary consumers (mollusks: mean $\delta^{15}\text{N}$ mollusk_{lake} = 11.6‰, mollusk_{river} = 13.0‰). Trophic position is based on a mean nitrogen trophic fractionation value ($\Delta^{15}\text{N}$) of 2.2‰ (McCutchan et al. 2003)

Population genetics- Microsatellite analysis. All our microsatellites followed Hardy-Weinberg equilibrium in both habitats and sampling periods. Observed and expected heterozygosity, the number of alleles per locus and F_{IS} are reported in table 2. After sequential Bonferroni corrections, only a single test of linkage disequilibrium was found to be significant, in the river population between STN 32 and STN75. There was no difference between number of alleles between populations (2 sampling points in 2 habitats) ($F_{3,129}=1.29$, $p=0.280$) and thus between habitats ($F_{1,129}=1.25$, $p=0.265$). An analysis of similarity (ANOSIM) revealed that the subsets of alleles differed between ecotypes ($R=0.458$, $p<0.001$). We found 45.8% of private alleles in the lake and 28.3% in the river. Wright statistics showed strong genetic differentiation between the two ecotypes (Mean F_{ST} +/- S.E, $F_{ST}=0.159$ +/- 0.0035, $p<0.001$) but not between sampling points (river: $F_{ST}=-0.0006$, $p=0.978$, lake: $F_{ST}=-0.0043$, $p=0.789$). Individual heterozygosity did not vary between populations ($F_{3,129}=0.909$, $p=0.439$) nor between habitats ($F_{3,129}=0.827$, $p=0.365$).

Using an individual-based cluster analysis, we found two clusters as optimal partitions confirming the correlation with habitat type (Reusch et al. 2001b). Moreover, all fish were reliably classified within their original population (Figure 2a).

Table 2: Genetic data analysis of river and lake populations. Habitat type, locus, sample size (N), mean allele number (N_A), heterozygosity (H) observed and expected, F_{is} and p values from Hardy-Weinberg test for heterozygote deficits across all fish within habitats.

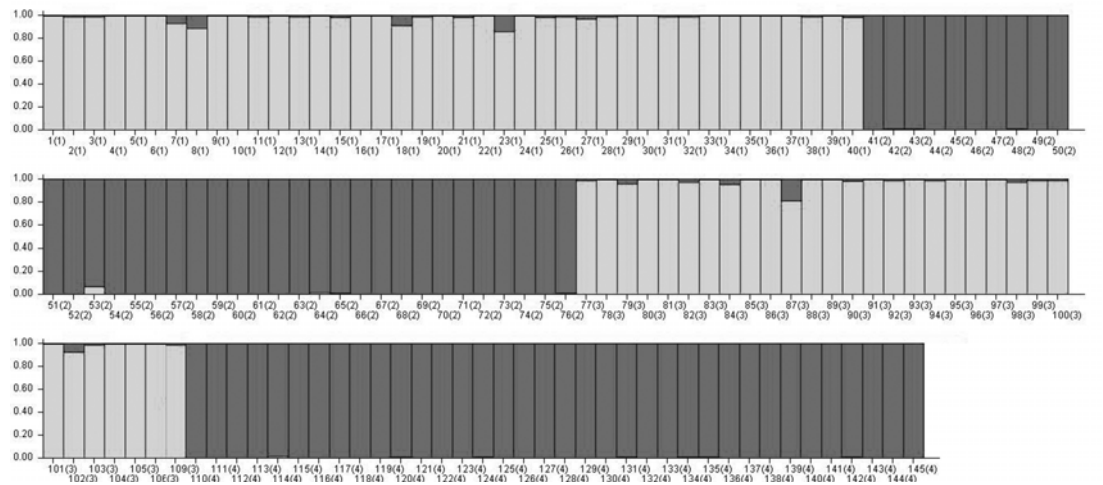
LAKE						
Locus	N	N_A	H_{obs}	H_{exp}	F_{is}	p
Gac4170	62	8	51	50	-0.0136	0.5235
Gac1125	62	10	48	57	-0.0195	0.7161
Gac5196	62	7	43	44	+0.0183	0.2556
Gac1097	62	6	45	47	+0.0371	0.3455
Gac7033	62	8	40	45	+0.1156	0.1720
STN32	62	9	38	41	+0.0626	0.3853
STN18	62	5	35	39	+0.0986	0.5371
STN75	62	7	34	38	+0.1046	0.1206
STN84	62	7	41	38	-0.0881	0.2773
River						
Locus	N	N_A	H_{obs}	H_{exp}	F_{is}	p
Gac4170	62	8	48	49	+0.026	0.2581
Gac1125	62	7	55	56	+0.009	0.4783
Gac5196	62	6	57	57	+0.008	0.3429
Gac1097	62	4	36	42	+0.159	0.0363
Gac7033	62	5	40	43	+0.074	0.1386
STN32	62	5	48	46	-0.040	0.8574
STN18	62	2	29	24	-0.221	0.9915
STN75	62	7	40	46	+0.139	0.077
STN84	62	5	52	47	-0.109	0.957

Parasite load.

We recorded 17 different parasites species from 6 taxonomic groups: Protozoa (*Trichodina* sp. and *Apiosoma* sp.), Monogenea (*Gyrodactylus* sp.), Digenea (*Diplostomum* sp., *Apatemon cobitidis*, *Phyllodistomum folium*, *Cyathocotyle prussica*, *Echinochasmus* sp.), Cestoda (*Valipora campylancristrota*, *Paradilepis scolecina*, *Proteocephalus filicollis*, *Diphyllobothrium* sp.), Nematoda (*Anguillicoloides crassus*, *Camallanus lacustris*, *Contracaecum* sp., *Raphidascaris acus*) and glochidia, the parasitic larval stage of freshwater mussels (Mollusca).

- **Difference between sampling points**

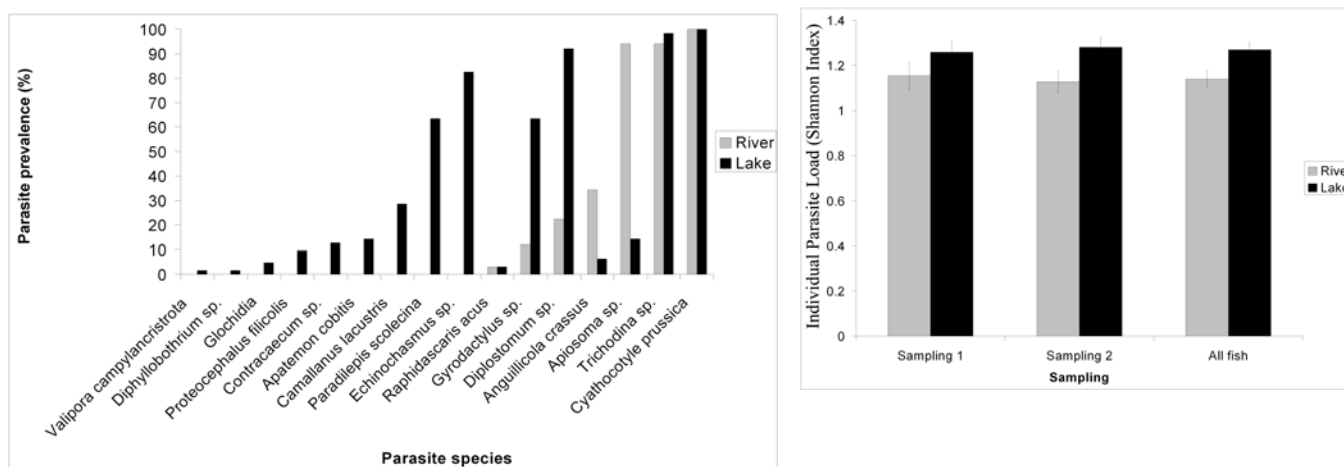
First, we tested whether the parasite load, calculated as the Shannon index, was different between sampling periods within a habitat. There was no difference between the fish sampled in January and those sampled in March (Lake, $U=448$, $p=0.52$; River, $U=555.5$, $p=0.83$: Figure 3a & b). Thus, we pooled data from the two sampling periods for each habitat to increase statistical power.



Chapter V Fig. 2: Barplot obtained from 6 independent runs of Structure 2.2 based on 9 microsatellites from 129 three-spined sticklebacks caught at 2 different sampling times in 2 different habitats (a river and a lake). Bars can be partitioned up to four fragments each standing for a potential populations. Fish caught in lake are referred to as 1,3 between brackets while river fish are referred to as 2,4 between brackets.

- **Difference between habitats**

Fish from the lake harbored a higher parasite load than fish from the river (t test, $t_{1,129}=-6.8736$, $p<0.0001$; Mean lake (\pm standard deviation) 1.10 ± 0.37 ; Mean river 0.70 ± 0.31 : Figure 3b). Furthermore, a community analysis revealed that fish from the two habitats harbored different parasite communities (Global $R=0.481$, $p<0.0001$), with an average dissimilarity of 60.9% between the two habitats. This difference was largely due to a ciliate, *Apiosoma sp.* and two trematodes *Diplostomum sp.* and *Echinochasmus sp.* that were common in lake sticklebacks: together these species contributed to 44.5% of the dissimilarity. Between-habitat differences remained when parasites were split into actively infecting vs. trophically transmitted parasites (active, Global $R=0.831$, $p<0.0001$; trophically transmitted, $R=0.334$, $p=0.0001$).



Chapter V Fig. 3: (a) Abundance of each parasite species split for the habitat in which the fish were caught (ordered for increase parasite prevalence in lake the in river fish). (b) Individual parasite load calculated as standardized Shannon index. The two different sampling points are represented and the average overall individuals (means and standard errors are depicted). In black the fish from lake, in grey those from river.

MHC analysis.

Transformations of individual MHC diversity values did not improve the data's approximation to a normal distribution, hence we conducted non-parametric tests. We detected 29 alleles in total, with 17 specific to lake fish, 5 to river and 7 were shared by fish from both habitats. The shared alleles displayed obvious frequency difference between habitats. On the total pools, variant frequencies ranged from 1 to 37.5% in lake and from 1 to 50.7% in the river population (Figure 4a). Individual MHC diversity was neither correlated to individual heterozygosity index based on microsatellites ($Rho=0.0404$, $p=0.667$) nor to the individual microsatellite diversity ($Rho=0.0047$, $p=0.960$).

- Differences between sampling points within habitat

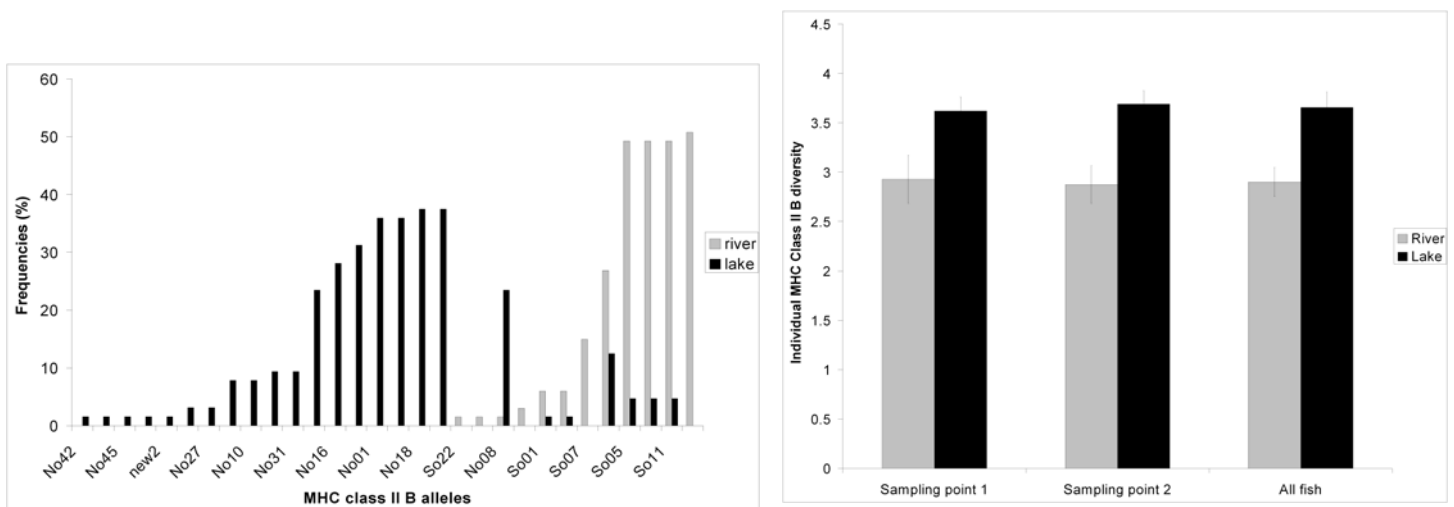
Neither the individual number of MHC variants (Mann Whitney, Lake, $U= 524$, $p=0.65$; River, $U=606$, $p=0.69$: Figure 4b) nor the pool constitution (ANOSIM, Lake, $Global R=-0.009$, $p=0.71$; River, $R=-0.019$, $p=0.82$) varied between sampling points. We therefore pooled data from the two sampling periods for subsequent between-habitat comparisons.

- **Differences between habitats**

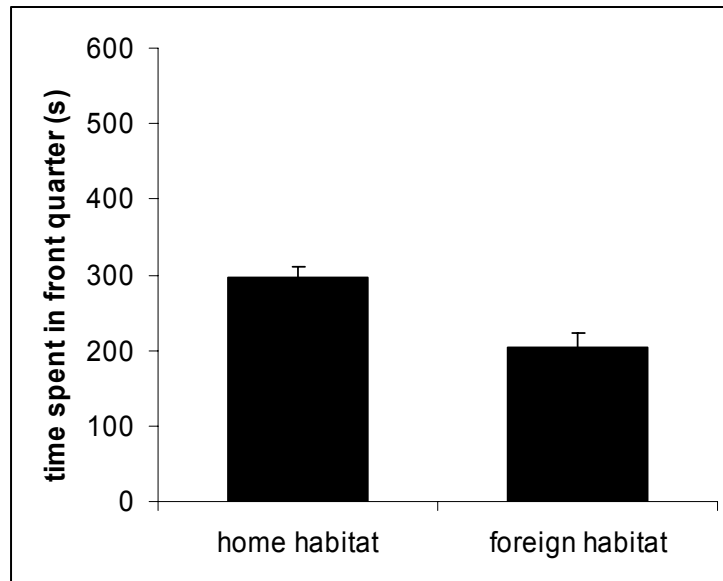
Fish from lake harbored a higher individual MHC diversity than river fish (Mann-Whitney $U=1462$, $p=0.0009$: Figure 4b). Moreover, the pool of MHC variants differed between the habitats (ANOSIM, Global $R=0.442$, $p=0.0001$). A SIMPER test revealed that the average dissimilarity between habitats was 97.1% and that 16 alleles contributed to 90% of this difference.

Mate choice. We tested whether females preferred the odor of males coming from the same habitat. Indeed, females significantly favored the side of the chamber associated with males originating from the same habitat (Binomial test, $p = 0.0013$). Moreover, we found that focal females spent significantly more time on the side of the males from the same environment (t-test, $t_{1,38} = 3.868$, $p= 0.0004$. Figure 5).

Splitting the dataset by female habitat of origin, our results revealed that lake females consistently preferred the lake male side (Binomial test, $p = 0.0107$) and spent as well more time on that side (t-test, $t_{1,18}= 3.602$, $p= 0.002$). River females chose the river male side significantly more often than the lake side (Binomial test, $p = 0.05$). River females demonstrated as well preference for the river males' side of the flow channel (t-test, $t_{1,18} = 2.126$, $p= 0.0476$).



Chapter V Fig. 4: (a) Distribution and frequencies of MHC class II B alleles found in lake and river fish, while (ordered for increase frequency in lake then in river population) (b) shows the higher individual MHC diversity in lake fish (although we used non parametric tests, means and standard errors are depicted). In black the fish from lake, in grey those from river.



Chapter V Fig. 5: Mean time + SE females spent in front quarter close to male water inflow when river and lake female choices are pooled ($t = 3.868$, d.f. = 38, $p < 0.0005$. Binomial test, $p = 0.0013$. See Appendix III for female choice with regard to female origin).

Discussion

Divergent natural selection acting on ecological traits, which also affect mate choice, is a key element of ecological speciation theory. However, because studies focused on species that have already undergone differentiation (e.g. Terai et al. 2006; e.g. Blais et al. 2007) or because genes responsible for variation at ecological and mating traits (“magic trait”) are rarely identified, it is difficult to pinpoint the putative mechanism underlying the divergence. To our knowledge, there is no study that used the same integrative approach as we did on the same dataset. Here, we investigated niche differentiation (using stable isotopes and parasite communities), divergence at adaptive and neutral loci (MHC and microsatellites), and female mate choice behavior. We have shown that three-spined sticklebacks from sympatric populations present strong morphological differences and specific niche utilization most likely as result of habitat adaptation. In relation to these adaptations fish from these populations harbored different parasite communities, and thus MHC allelic pools. Moreover, mate choice based on a genetic component of the olfactory cues showed assortative mating for habitat of origin. Here we identified links between mate choice and ecology which contribute to speciation.

Reproductive isolation between populations may evolve from adaptation to different environments (Coyne & Orr 2004; Dieckmann et al. 2004). When investigating an ecologically and genetically-determined phenotypic trait (lateral plate abundance), we detected strong

phenotypic divergence between habitats. Factors driving variation in plate morphology in sticklebacks have been the focus of intensive research (Bell & Foster 1994; Bourgeois et al. 1994; Cresko et al. 2004; Colosimo et al. 2005; Marchinko & Schluter 2007). Here, river fish were exclusively low-plated, whilst lake sticklebacks exhibited plate polymorphism ranging between low- to fully-plated. Lateral plates are thought to have followed adaptation to fresh water habitats after glacial retreat at the end of the Pleistocene. Lateral plates reflect different pressures such as predation, feeding behavior and availability of elementary ions that might have driven the morphs evolution towards a plate reduction (e.g. Bell & Haglund 1978; e.g. Reimchen 1992; Bourgeois et al. 1994; Bergstrom 2002). Our stream ecomorphs might thus be considered as the last step of plate reduction reflecting adaptation to the environment. Moreover, the discriminant analysis we performed showed strong morphological differences between ecomorphs, emphasizing as well habitat adaptation and the evolution of shape.

Parasites represent a strong selective pressure to which hosts have to adapt (Hamilton & Zuk 1982). Consistent with previous studies (Kalbe et al. 2002; Wegner et al. 2003b), we showed that fish from lake and river were infected with different parasite communities. The difference in parasite communities between habitats remained strong when dividing the data set into actively infecting vs. trophically transmitted parasites. This strongly suggests that the difference is not only due to ecosystem differences, that in term support different parasite taxa, but also to differences in stickleback trophic ecology. Analysis of stable isotopes revealed lake and river sticklebacks inhabited distinct areas of $\delta^{13}\text{C}$ - $\delta^{15}\text{N}$ isotope space, indicating marked ecological differences between the two habitats. Furthermore, lake fish displayed an increased trophic niche width ($\delta^{15}\text{N}$) than their river counterparts, feeding at a greater range of TPs (2.7-4.4) compared to river sticklebacks (3.3-4.3). Lake fish also showed more variation in $\delta^{13}\text{C}$ values reflecting the availability of benthic and pelagic food sources, compared to the river, where pelagic sources of energy are absent. Exposure to a greater variety of putative food resources may therefore drive exposure to an increased range of parasites, both trophically, and actively-transmitted in the lake stickleback ecomorphs.

As parasites are thought to be the prime source of the evolution of the MHC (Klein et al. 1993), it is not surprising to observe different MHC variants between divergent habitats (Summers et al. 2003; Blais et al. 2007; Ekblom et al. 2007; Eizaguirre et al. 2008a). We found that under contrasting parasite pressures, fish populations displayed different pools of MHC variants, both in constitution and in frequencies. This is the first time that, in addition to MHC diversity (Wegner et al. 2003b), allelic pools of MHC class II B, between ecotypes are investigated in sticklebacks. Differences in MHC allelic pools between populations (and not species) has been

proposed but the selective contrasting macroparasite communities has seldom been investigated.

When compared to the entire allelic population, intra-individual MHC diversity (defined as the overall number of different classical MHC alleles at all loci) is limited to a subset of alleles. From humans to salmonid fishes, the limited number of loci prevents the entire potential MHC allele pool to be displayed (Lawlor et al. 1990; Janeway et al. 2001; Robinson et al. 2003). Thus, differential parasite-driven selection between habitats may favor some alleles and disfavor others. This eventually confers the two host populations with different allele pools allowing the formation of distinct species (Ridley 2003) through the pleiotropic role of MHC genes in immunity and mate choice (Eizaguirre et al. 2008a). For instance, a study investigating local resistance to malaria in two house sparrow populations provided evidence for population-specific MHC controlled resistance to the pathogen (Bonneaud et al. 2006b). Such prediction should hold true for all MHC-locally adapted vertebrates.

Different pools of MHC alleles do not necessarily define species. Indeed, trans-species polymorphism model predicts allele sharing even between genetically distant species (Klein et al. 1993). MHC adaptation can drive and accelerate population divergence, which should be reflected genome wide. In the present study, genetic divergence between ecotypes was observed not only in adaptive genetic loci (MHC) but also on microsatellites, revealing different history of the populations. Such finding were obtained on a larger scale on populations from the same drainage system (Reusch et al. 2001b), what reinforces the generality of our conclusions.

Neither the individual-based genetic analysis nor stable isotopes assays detected potential migrants: this might be interpreted as the consequence of reduced fitness in foreign habitat. This is congruent with a study in three-spined sticklebacks from neighbor populations where lab-bred fish from pure F2 lake lines were not able to grow as well as F2 river lines when exposed to river conditions (Scharsack et al. 2007). The reduction in growth could potentially be attributed to a trade-off between growth and immune system due to maladaptation to local parasite fauna (Scharsack et al. 2007; Eizaguirre et al. submitted b). Such pressure would surely prevent lake fish from migrating to river habitat, further reducing gene flow between the habitats. Lakes presenting higher diversity and intensity of parasite species (Kalbe et al. 2002; Wegner et al. 2003b), might not be suitable for MHC reduced diversity of river fish- again reducing gene flow between the populations.

Additionally, the individual-based genetic analysis did not reveal the presence of obvious hybrids either. Several studies have tried to explain lower hybrid fitness under natural conditions in sticklebacks (Hatfield & Schluter 1999; Kalbe & Kurtz 2006; Gow et al. 2007) as well as in mice (Sage et al. 1986) and parasite reduced fitness has often been invoked. We

propose that river-lake hybrids, originating from disassortative mating, might suffer higher parasitization due to lack of locally-adapted MHC variants and/or stronger T cell depletion due to super-optimal number of MHC alleles (Milinski 2006; Eizaguirre et al. 2008a). Surprisingly, as discussed in their study Rauch *et al.* (2006b) found a relative low effect of local MHC genotype, maybe due to a too short time period of exposure to a low parasite pressure.

To be relevant in an evolutionary perspective, the different adaptations individuals have undergone should be reflected through mate choice. Here, we report evidence for olfactory mediated reproductive isolation between three-spined sticklebacks from different, but adjacent habitats. Using water taken directly from above male's nests, females preferentially chose those from the same habitat. The nest has been demonstrated to be central for signaling MHC and male factors (Milinski et al. 2005; Sommerfeld et al. 2008). This emphasizes once more the role of olfactory cues in mate signaling and adds to the list of evidence for odor-mediated mate choice (see for review Penn 2002; see for review Milinski 2006). Assortative mating according to ecomorph has been reported in a handful of studies (e.g. in beak of Darwin's finches Huber et al. 2007). Boughman *et al.* (2005) and McKinnon *et al.* (2004) have shown limnetic/benthic pairs of sticklebacks to be reproductively isolated with fish size being of prime importance. Later, Vines & Schluter (2006) reported similar results with allopatric lake-stream stickleback populations. We argue here that wild-caught fish and those fish exposed to natural conditions could be signaling information regarding parasitization and thus MHC. Rafferty *et al.* (2006) made the same genetic assumption showing assortative mating based on olfactory cues in sympatric benthic/ limnetic stickleback species. After habitat translocation, it was suggested that shoaling behavior in stickleback was reversible within 3-4h based on diet affecting odors (Ward et al. 2007). Our finding points more towards a genetic component of the olfactory cues: the first tests were performed 10 days after catching and the fish were fed under common diet in the lab. Thus, we suggest that the contrasting parasite communities the fish from lake and river have to face selected for different MHC alleles, which are reflected in odor differences.

It is worth noticing that theoretical work has predicted a purifying effect of assortative mating (Kirkpatrick & Nuismer 2004). However, this might not apply to MHC genes since allele frequencies could vary rapidly between years responding to fluctuating parasite pressures, as expected under negative-frequency-dependent selection (e.g. Wegner 2004; Westerdahl et al. 2004; Charbonnel & Pemberton 2005). This is especially true when individual heterozygosity is not related to MHC diversity.

Premating isolation between limnetic/benthic stickleback ecomorphs was suggested to arise initially as a by-product of divergent natural selection on key traits and was later reinforced in

sympatry (Rundle et al. 2000). Our results seem consistent with this prediction. During recolonization, niche specific utilization might have led to ecological specialization, which in turn might have led to the development and maintenance of separate host-parasite arms races in the different ecomorphs. However, MHC mediated niche utilization with selection acting on mal-adapted individual with regard to parasite resistance appears as well as a potential scenario.

Few studies of vertebrates have identified genes acting on an ecological trait and affecting mate choice. Cichlids from the great African Lakes represented so far a unique model to study speciation. Terai *et al.* (2006) have shown that divergent selection fixed opsin proteins with different predicted light absorbance properties adapting populations of cichlids to light environment. Divergent evolution of the visual system coincided with male nuptial coloration- a determinant feature for assortative mating in cichlids. In cichlids parasite mediated speciation has been proposed (Blais et al. 2007) and mate choice based on olfactory cues was confirmed (Plenderleith et al. 2005). However, since the species studied have already been through differentiation distinguishing causes from consequences was not possible.

In summary, using a multidisciplinary approach, we show clear morphological and niche utilization differences between lake and river morphs. Linked to habitat adaptation fish are exposed to contrasting parasite communities and thus display different MHC allele pools. Assortative mating exerts a pre-zygotic isolation and enhances the pace of population separation. Due to maladapted individual MHC diversity in hybrids and an associated higher parasite load, it is likely that post-zygotic selection acts against hybrids. Hence, MHC pleiotropic effects can induce and maintain reproductive isolation between populations, even in the absence of geographical boundaries and can be regarded as the “magic trait”.

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Chapter VI

MHC local adaptation: a field experiment

Christophe Eizaguirre & Tobias L. Lenz contributed equally to this study.

Co-workers: Chris Harrod, Martin Kalbe, Manfred Milinski.

Abstract

The polymorphic genes of the major histocompatibility complex (MHC) have been proposed to enable individuals to adapt and therefore survive and reproduce in their local pathogenic environment. However, the presence of local MHC adaptation has so far not been experimentally tested. For the present study, we crossed three-spined sticklebacks from a river and a lake habitat displaying almost totally distinct MHC allele pools. Intercrossing F1 hybrids, we obtained a second fish generation segregating into pure river, pure lake and two hybrid MHC genotypes, while at the same time randomized for the genetic background. We used a double common garden experiment where fish were exposed during 10 months simultaneously to lake and to river conditions using replicated cages. We found that MHC genotype was correlated to parasite load in hybrid lines. The total parasite load harbored by the fish appears to result from an adaptive interaction between the innate and the acquired immune system of the hosts, in turn driven by different parasite strategies. Generalist parasites were more successful in river fish lines, which naturally encounter low pressure from these parasites. However, fish bearing lake MHC genotypes were more infected than fish carrying river MHC genotypes, when confronted with a stickleback-specific parasite, which is commonly present in river. In addition, we found support for additive and synergistic role of the genetic background and innate immunity in parasite resistance. In conclusion, we unravel experimentally the effects of two locally adapted genomes in general and MHC allele pools in particular and provide evidence for their interaction with contrasting parasite strategies.

Introduction

The major histocompatibility complex (MHC) represents a gene dense genomic region and displays some of the most polymorphic loci in the vertebrate genome (Klein 1986; Apanius et al. 1997). It consists of genes coding for cell surface molecules that present self and non-self peptides to T-cells and therefore play a key role in the onset and modulation of the specific immune response (Klein 1986; Janeway et al. 2001). Due to their tight linkage with the immune system, the evolution of MHC genes is thought to be under parasite driven selection (Klein 1986; Potts et al. 1994; Apanius et al. 1997). MHC genes generally present a high intra- and inter-individual polymorphism. Such polymorphism is thought to enable individuals to fight against the spectrum of parasites they are confronted with in their local environment (Apanius et al. 1997; Bernatchez & Landry 2003; Piertney & Oliver 2006).

In a local environment, ecological conditions, i.e. biotic and abiotic factors, but also community structure are likely to influence the presence and abundance of certain intermediate hosts, which are in turn necessary for the maintenance of different parasite taxa (Thompson 1994). Therefore, contrasting environments are likely to support distinct parasite communities, which would then require contrasting immunogenetic adaptation of hosts as well. This is supported by recent studies investigating MHC variation in neighboring populations, which showed that habitat heterogeneity can shape MHC composition, probably due to contrasting parasite communities (Wegner et al. 2003b; Bonneaud et al. 2006b; Blais et al. 2007; Ekblom et al. 2007; Alcaide et al. 2008; Babik et al. 2008). However, often the parasite side of these studies has not been revealed.

It has been proposed that parasitic exploitation could have the potential to promote speciation by low hybrid fitness relative to specifically co-evolved local host genotypes (Haldane 1992; Turelli et al. 2001). Although a variety of theoretical models have provided support for this idea (e.g. Maynard Smith 1966; e.g. Rice 1987) it remains controversial. The “Red Queen hypothesis” postulates that interactions among species, e.g. hosts and parasites, lead to constant natural selection for adaptation and counter-adaptation (Van Valen 1973). Usually, in a host-parasite arms race, parasites are expected to have an advantage over the host and to trade off virulence and host mortality (Ebert 1994). In addition, studies have reported that local adaptations resulted from parasites tracking locally common host genotypes and thus meeting a necessary condition of the Red Queen hypothesis (Ebert 1994; Lively & Dybdahl 2000). Local adaptation is therefore defined as higher parasite fitness on a sympatric than on an allopatric host (Ebert 1994). Interestingly, a model (Kawecki 1998), a meta-analysis (Lajeunesse & Forbes 2002) and an experimental test (Kalbe & Kurtz 2006) point towards a more complex theory involving different parasite strategies. If parasites and hosts are engaged in a co-evolutionary process, then specialist parasite lineages will have an advantage over

generalist parasites on a specific host genotype (Lajeunesse & Forbes 2002). As specialist parasites need to counteract evolution of host resistance, because they cannot switch to another host, they have to evolve faster in response to the host reaction, compared to generalist parasites. Such arms races should result in lower parasite burden from generalist parasites and higher infection from specialist parasites in local host genotypes. Since individual hosts are generally exposed to a broad variety of pathogens, an optimal host response that counteracts both threats should be expected.

In addition, it was recently proposed that the polymorphic genes of the major histocompatibility (MHC) could act as pleiotropic genes adapting individuals to their local pathogenic environment and leading to assortative mating, and therefore could facilitate speciation (Blais et al. 2007; Eizaguirre et al. 2008a). However, correlative evidence is scarce and thus the scientific community awaits empirical tests. In the present study, we tested the specific role of MHC genes in host adaptation to local parasite fauna- a pre-requisite to MHC pleiotropism. For this purpose, the three-spined stickleback (*Gasterosteus aculeatus*) represents an exceptional study organism. In northern Germany, populations colonized lakes and rivers after the last glaciation (Reusch et al. 2001b). Interestingly, these populations experience practically no gene flow (Reusch et al. 2001b), are confronted with contrasting parasite communities (Kalbe et al. 2002; Wegner et al. 2003b) and display low overlapping MHC allele pools (Eizaguirre et al. Submitted a). These characteristics led us to a specific breeding design, combining the effect of segregation and genetic mechanisms to produce hybrid lines with randomized genetic background. This in turn enabled us to focus on the MHC genotype effect without confounding background (Rauch et al. 2006b). Using F₂ fish, we performed a double common garden experiment. We simultaneously and reciprocally exposed these hybrid and pure line F₂ fish to the lake and the river pathogenic environments, where their parental generation had been caught. Here, we aim at experimentally showing that host MHC genes are adapted to the sympatric local parasites, which would explain the observed olfactory based assortative mating within these exact two populations (Eizaguirre et al. Submitted a) by maintaining local MHC adaptation.

Material & Method

In this section we will describe the methods used for the full experiment with a large integrative approach. However, for the results we will keep a major focus on parasite load and MHC.

Breeding design. We sampled three-spined sticklebacks (*Gasterosteus aculeatus*) from two habitats, a small, slow-flowing river (Malenter Au, 54°12'16.19"N, 10°33'32.93"E, Germany)

and a lake (Großer Plöner See, 54° 9'21.16"N, 10°25'50.14"E, Germany) in spring 2006. Fish were kept singly in 16L tanks with constantly flowing water. In June 2006, the fish were transferred to summer condition (day light 14h, T 18°C) in order to reach sexual maturity. Males were provided with artificial nesting material (sand and nylon threads). We crossed fish randomly with respect to fish identity, but not to fish origin, in order to obtain lab-bred sibships with pure lake, pure river and hybrid genomic background (see fig 1.). For breeding males and females were allowed to display natural behaviors and females spawned into the male's nest. Clutches were left for one hour in the nest after fertilization before being taken out. Individual egg clutches were incubated in aerated well water (with 0.04 ppm malachite green) at 18°C, until hatching. We obtained 40 individual clutches with the three following backgrounds: pure lake (10 crosses between lake fish), pure river (10 crosses between river fish) and hybrids (20 crosses between a lake and a river fish). As the same fish were used in both pure and hybrid crosses, to assure the same genetic material in the different lines, 11 of the hybrids had a mother of lake origin and 9 had river mothers. The F1 generation was progressively transferred to autumn, winter, and spring conditions, from when on the fish were kept singly. Finally they were brought to summer condition where the males were provided with nesting material. Following the same protocol, we randomly interbred within hybrid lines and within pure lines in order to get the second fish generation (F2). From the initial 40 F₁ crosses, we therefore obtained 20 F₂ families with three different genomic backgrounds: 5 river, 5 lake, 10 hybrid. With this design, we aimed at producing hybrid families with randomized genetic background, which could then be analyzed for origin specific MHC genotype effect.

In each F2 hybrid family, four different MHC genotypes can be expected: RR, LL, RL and LR. The first letter stands for the haplotype inherited from the F1 father, the second letter for the haplotype inherited from the F1 mother. L and R represent the lake or river origin of the haplotype respectively. Original lake and river population diverging strongly in their MHC allele pools, this design should result in F2 offspring segregating within each family into four distinct MHC genotypes with a shared randomized genomic background. Using F2 fish eliminates as well any potential maternal effects.

Field exposure. On September 3, 2007, fish were brought to the natal place of the parental generation (the river Malenter Au, 54°12'16.19"N, 10°33'32.93"E and the lake Großer Plöner See, 54° 9'21.16"N, 10°25'50.14"E, Germany). We stocked 38 cages with the 10 hybrid families. Each cage contained 20 fish, partitioned as follows: 10 random fish from one hybrid family, plus one fish of each pure family (5 river and 5 lake). For the hybrid families we created 4 replicates (except for one family where we achieved only 2 replicates). 2 (1) cages per family were located into the lake and the 2 (1) others were placed into the river.

In February, before the fish reached sexual maturity, the sexes were separated with a fine mesh net to avoid potential breeding.

The fish stayed in the cages for 10 months from September to June and collection started on the 5.06.2008. In the middle of this period a cage was destroyed in the river after a thunderstorm. Once a week, all cages were checked for dead fish and the water temperature, oxygen content and conductivity were measured.

The cages were long and flat (1m x 0.25m x 0.6m) in order to assure constant emergence even in the shallow river. They consisted of stainless steel mesh and a stable framework. The 5 mm mesh allowed only small particles and most invertebrates (food items and intermediate hosts of various parasite species) to pass through. We placed 19 cages at an interval of 3 meters in the center of the river bed (about ca. 3 m wide and 1.5m to 0.5 m deep). The other half of the replicates was placed in the shallow part of the lake at about 1-1.2 m depth at 2 m intervals.

MHC typing. The MHC class IIB diversity was determined by reference strand conformation analysis (RSCA) according to Lenz *et al.* (in prep). We amplified the exon 2 of MHC class IIB genes that encodes for the peptide-binding groove. This exon has previously been suggested, to be under parasite mediated positive selection as revealed by a high ratio of nonsynonymous over synonymous substitutions (d_N/d_S) (Reusch *et al.* 2004). The MHC loci in the stickleback are duplicated and the number of sequence variants varies substantially between individuals (Wegner *et al.* 2003a); (Reusch & Langefors 2005), Lenz *et al.* in prep). For simplicity we will refer to the different sequence variants as alleles, although they may stem from different loci.

Microsatellite typing. For each F2 fish, DNA extractions from dorsal spine were performed using the Invisorb DNA Tissue HTS 96 Kit / R (Invitex, Germany) on an automated platform (Tecan, Switzerland) following the manufacturer's protocol. All fish were typed for 9 microsatellites combined in 2 different PCR multiplex protocols before and after the experiment (Reusch *et al.* 2001a; Kalbe *et al.* submitted). We used these loci to identify fish at the end of the experiment.

Parasite load. Since the number of fish was too high to be handled within three days as aimed at in previous studies (Kalbe *et al.* 2002; Kalbe *et al.* submitted), dissection took place with regard to cage replicates (80 fish) to keep all MHC genotype comparisons possible. Since cages were half stocked with hybrids and half with pure lines, pure and hybrids lines are directly comparable. The dissections were spread over four weeks and the total number of fish recovered was 714. All external and internal macroparasites and ciliates were determined to the lowest taxonomic level possible using dissection microscopes (for dissection protocol, see Kalbe *et al.* 2002).

In order to quantify the total parasite load of each fish, the Shannon index, which accounts for both abundance and evenness of the present species, was calculated following a fourth root transformation of parasite abundance. This allowed us to summarize different combinations of parasites for each stickleback and to make a quantitative comparison of their total parasite burden. We calculated a second Shannon index taking only generalist parasites into account. As for the specialist parasites, since *Gyrodactylus sp.* was the only one with a sufficient abundance (more than 1 %), we will focus on it. During dissection fish mass (± 0.1 mg), standard length (± 1 mm) and lateral plate number were recorded.

Immune assays. For immunological assays, leucocytes were isolated from head kidney (HKL). Head kidney preparations followed Scharsack *et al.*'s protocol (2004). Total cell numbers in HKL isolates were determined with the standard cell dilution assay. Additionally, flow cytometric measurements of freshly isolated HKL were used to determine proportions of granulocytes (FSC/SSC-high) and lymphocytes (FSC/SSC-low) in individual HKL samples (all according to Scharsack *et al.* (2007)).

As an important factor of the cell mediated innate immunity, the respiratory burst activity of HKL was quantified (Kurtz *et al.* 2004). As a parameter for activation of the adaptive immune system, frequencies of lymphocytes in G0-1, S and G2-M phases were acquired by DNA content analysis of red fluorescence intensity (propidium iodide labelling) of single cells from the lymphocyte gate (Scharsack *et al.* 2007) .

MHC expression. As gene expression is a good diagnostic tool of the current physiological state of an individual and reflects the need for the final product encoded by the target gene, we performed quantitative real-time polymerase chain reaction (qRT-PCR) to follow MHC gene expression in the fish.

RNA was extracted from spleens, known as an immuno-relevant organ. The spleen is a major secondary lymphoid organ of teleost fish which is mainly involved in blood filtration, antigen trapping and processing. Immediately after killing and opening the fish, the spleens were stored in RNA later and kept at -20°C until RNA extraction. The RNA was extracted with the NucleoSpin 96 RNA kit (Macherey Nagel, Düren, Germany) following the manufacturer's protocol. Organs were homogenized in 1% mercapto-ethanol and buffer in a Retsch mill by shaking samples 3 minutes at 30Hz. Elution was performed in two steps in a final volume of 100 μl . Reverse transcription was carried out with Omniscript Reverse Transcriptase kit (QIAGEN). As gene expression is a relative value, the L13A gene coding for the ribosomal binding protein was used as house keeping gene following Hibbeler *et al.*'s (2008) advices. Housekeeping genes serve as internal control for reverse transcription and allow for relative quantification of the target gene. qRT-PCR was performed following Wegner *et al.*'s (2006)

protocol developed for MHC class II genes in the three-spined stickleback. For each individual, three qRT-PCR runs were performed and values were averaged to counteract potential run error. We calculated average Δ CT values by subtracting mean CT of the target gene to the mean CT value of the internal control for each individual.

Stable isotopes. It has been proposed that fish originating from the same populations as the ones used in the current study could be using habitat resources in a different manner (foraging behavior, physiological processing of the food) and habitat use could be linked to MHC genotype and parasitization patterns (Eizaguirre et al. Submitted a). Using a common garden experiment as the one reported here, allows for directly comparing food use by the fish with different genetic backgrounds (pure lake, pure river, and hybrids) and MHC genotypes. Previous studies have used stable isotope analysis (SIA) to reveal individual migration between ecologically contrasting but connected habitats in aquatic consumer species as diverse as European eels (Harrod et al. 2005) and Galápagos sea lions (Wolf et al. 2008). SIA have been used as well to report niche use and are an exceptional tool to describe the trophic level of an organism. During dissection, a section of caudal muscle was dissected, and oven dried for 24 hours at 65 °C. Samples were grinded, weighed in tin cups and analyzed for carbon ($\delta^{13}\text{C}$) and nitrogen ($\delta^{15}\text{N}$) stable isotope ratios and elemental C and N composition (see for details Harrod et al. 2005). Repeated analyses of internal standards showed precision of < 0.1 ‰ (carbon) and < 0.3 ‰ (nitrogen). $\delta^{13}\text{C}$ data were arithmetically lipid-normalized following Kiljunen *et al.* (2006).

Statistical analysis. Difference in parasite communities between habitats was tested with an ANOSIM (Analysis of similarity) based on a Bray-Curtis similarity matrix.

We estimated the importance of MHC genotypes in parasite resistance in the hybrid lines by nesting the MHC genotypes within the family effect in an analysis of variance (ANOVA). The dependent variable represented the residuals of a two-way ANOVA between parasite load, habitat and sex to control for both effects. For all the tests we controlled for family effects treating it as random factor. Such analysis was not only conducted on the total parasite load but as well on a specialist stickleback parasite, the trematode *Gyrodactylus sp.*, and on a Shannon parasite index including only the generalist parasites (Table 2).

To test for local adaptation in the hybrid families, we determined the interaction between origin of MHC (LL or RR) and the habitat. We tested as well a general local adaptation by estimating the interaction between breeding origin (pure lake, pure river and hybrid) and the habitat of exposure. For these tests, we used a split plot design and controlled for family effect.

Statistical analyses were conducted using R statistical package (v. 2.5.0) for Windows XP and Primer v6 (Clarke & Gorley 2006). Normality of distributions and variance homoscedasticity were verified and tests

conducted accordingly. If necessary, dependent variables were log or square root transformed to meet tests' assumptions.

Results

MHC genotypes. In total we detected 28 different MHC alleles. We did not observe any event of recombination. In all hybrid lines but one, we could clearly determine the population origin of the different MHC genotypes. This one family was excluded from all analyses due to origin-overlapping alleles in the parental genotypes. The following results were thus obtained from nine hybrid families, five pure lake and five pure river families. In total, we detected 19 alleles in the lake pure lines, 15 in the pure river and 22 in the hybrid lines (Fig. 1). Descriptive information of MHC diversity is given in table 1. One F2 hybrid family showed MHC homozygous individuals due to the presence of the same haplotype (allele No05) in both F1 crosses.

In general, homozygous F2 fish displayed a variable number of MHC variants, ranging from 1 to 3. This suggests either that in some haplotypes the same variant is present on different loci or that individuals display a variable number of MHC loci. This variation between haplotypes could be followed and was consistent over the three fish generation (Parents, F1, F2).

Parasite load

- *Comparison between habitats*

We recorded a total of 24 different parasite species (Table 2). 22 were found in fish exposed to the lake, whereas 14 were counted in fish exposed to the river. Among these parasite species, 10 were lake-specific, whilst only 2 were found only in fish exposed to river conditions (Fig. 3). The detected parasites differed in their infection and transmission strategies (Table 2).

Parasite communities found on the fish differed between both habitat of exposure (ANOSIM, $R=0.625$, $p<0.001$). A similarity percentage analysis of species contributions revealed that the average dissimilarity between parasites communities was 50.60%. Such habitat differences remained when considering only actively infecting parasites (ANOSIM, $R=0.528$, $p<0.001$). The difference was mainly driven by the intensity of the specialist *Gyrodactylus sp.* in river and the generalists *Apatemon cobitis* and *Diplostomum sp.* in the lake. This suggests that the river parasite community is driven by specialist parasites, whereas generalist parasites might dominate the lake parasite community. Trophically transmitted parasite communities were as well different between habitats (ANOSIM, $R=0.087$, $p=0.001$), and the variation was mainly due to a higher abundance of *Raphidascaris acus* in river and *Camallanus lacustris* in lake.

Chapter VI Table 1: Description of MHC diversity in fish exposed to natural lake and river conditions.

A) ALL FISH	NUMBER OF INDIVIDUALS	MEDIAN MHC DIVERSITY	RANG OF MHC DIVERSITY	MHC HETEROZYGOSITY
Background				
Pure Lake	176	4	2-5	0.97
Hybrids	349	4	1-5	0.898
Pure River	173	4	1-5	0.591
b) Hybrid fish	Number of individuals	Median MHC diversity	Ranged MHC diversity	MHC Heterozygosity
MHC genotype				
LL	50	4	3-4	1.00
LR	53	5	1-5	0.848
RL	96	3	3-5	1.00
RR	99	4	1-5	0.68

- MHC genotype

Using the F2 hybrids for which we could determine the origin of the MHC genotype, we tested whether the parasite load was correlated with these genotypes and with the family once corrected for habitat and sex differences. We found a strong family effect ($F_{8,263}=6.048$, $p<0.001$. Fig. 2a) and a significant MHC genotype effect ($F_{26,263}=1.520$, $p=0.050$. Fig. 2b).

Interestingly, this effect of the MHC genotype remained when considering a specialist parasite like *Gyrodactylus sp.* ($F_{26,263}=1.561$, $p=0.044$), but not when focusing on the index of generalist parasites ($F_{26,263}=0.646$, $p=0.909$). Consistent with the results obtained with the general parasite load index, in both cases the family effect was pronounced ($p<0.001$)

- MHC local adaptation

Expectedly, parasite load (Shannon index) was higher in the lake than in the river habitat ($F_{1,263}=84.226$, $p<0.001$). We found evidence for local adaptation in the hybrids as seen in the significant interaction between habitat of exposure and origin of MHC ($F_{3,263}=3.226$, $p=0.031$. Fig. 3a). Fish with an LL MHC genotype displayed a higher parasite load than RR MHC fish in lake. The opposite was observable in fish exposed to river conditions.

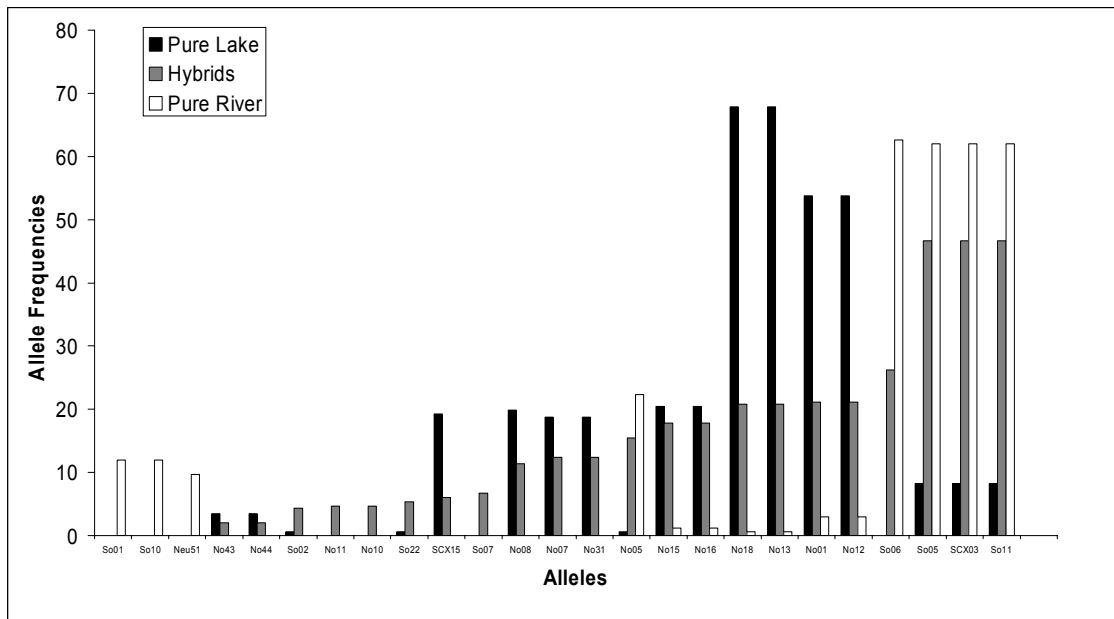
When focusing on generalist parasites, we found a habitat ($F_{1,263}=182.678$, $p<0.001$. Fig. 3b) as well as a family ($F_{8,263}=3.479$, $p<0.001$) effect, but no correlation with MHC genotypes ($F_{3,263}=0.958$, $p=0.413$) and no interaction of MHC and habitat. In contrary, investigating the specialist *Gyrodactylus sp.* revealed an MHC genotype effect ($F_{3,263}=3.668$, $p=0.012$. Fig. 3c): in both habitats fish displaying LL MHC genotype harbored a higher number of *Gyrodactylus sp.*

Chapter VI Table 2: Table summarizing the prevalence (percentage of infected hosts), the mean intensity (number of parasite individuals of a given species per infected host), and the maximum number of parasite of all recorded parasites in the 714 dissected fish.

Parasite species	Parasite strategy	Transmission	Lake			River		
			Mean	Max.	Prevalence	Mean	Max.	Prevalence
Diplostomum sp.	Generalist	Active	18.04	68	99.18	16.88	75	97.14
Apatemon cobitis	Generalist	Active	9.22	296	86.30	0.23	6	11.14
Trichodina sp.	Generalist	Active	9.09	100	83.01	34.43	100	95.43
Glochidia	Generalist	Active	5.53	92	76.71	0.06	14	1.14
Gyrodactylus sp.	Specialist	Active	12.84	248	72.33	81.06	1073	99.43
Apiosoma sp	Generalist	Active	4.64	100	58.63	33.86	100	69.14
Cyathocotyle prussica	Generalist	Active	1.20	37	44.38	4.13	21	85.71
Echinochasmus sp.	Generalist	Active	0.73	7	40.55	0.02	2	1.14
Argulus foliaceus	Generalist	Active	0.84	16	38.63	0	0	0
Camallanus lacustris	Generalist	Trophic	0.34	4	24.93	0	0	0
Trematode X	Generalist	Trophic	0.33	8	18.36	0	0	0
Tylodelphis calvata	Generalist	Active	0.37	25	15.34	0	0	0
Raphidascaris acus	Generalist	Trophic	0.16	3	13.15	0.42	4	29.14
Contraecaecum sp.	Generalist	Trophic	0.09	4	7.12	0	0	0
Paradilepis scolecina	Generalist	Trophic	0.07	2	5.48	0	0	0
Anguillicoloides crassus	Generalist	Trophic	0.05	1	4.93	0.05	2	5.14
Proteocephalus filicollis	Specialist	Trophic	0.05	2	4.38	0	0	0
Phyllodistomum folium	Generalist	Trophic	0.04	2	2.74	0	0	0
Valipora campylancristrota	Generalist	Trophic	0.03	4	1.37	0	0	0
Ichthyophthirius multifiliis	Generalist	Active	0.01	1	1.10	0.02	1	1.43
Ergasilus sp.	Generalist	Active	0.01	1	0.82	0	0	0
Nematode X	Unknown	Trophic	0.01	1	0.55	0	0	0
Cestode X	Unknown	Trophic	0.00	1	0.27	0	0	0
Piscicola	Generalist	Active	0.00	1	0.27	0.01	1	0.86
Acanthocephalus lucii	Generalist	Trophic	0	0	0	0.06	2	6
Metacercaria X	Unknown	Active	0	0	0	0.01	2	0.86

X is used for parasites currently under determination.

Chapter VI Fig. 1: Graph presenting allele frequencies obtained in the F2 families with regard to breeding origin.



- Background genome local adaptation

In the test for background genome adaptation using all the fish, we found a strong significant interaction between habitat of exposure and breeding origin (pure lake, pure river and hybrids) ($F_{2,629}=16.166$, $p<0.001$). Pure lake background fish displayed a higher parasite load in lake than pure river background fish, and vice versa in the river habitat where pure river lines were more infected. Hybrid fish stand at an intermediate level (Fig 4). We did not find a significant interaction between origin and habitat of exposure in generalist parasites ($F_{2,629}=0.831$, $p=0.4363$) but instead both variables alone predicted parasitization (Habitat: $F_{1,629}=328.364$, $p<0.001$; Origin: $F_{2,629}=17.201$, $p<0.001$). Fish exposed to the lake habitat harbored a higher parasite load than fish exposed to river conditions. In addition, fish with a pure lake genome showed a consistently lower parasite load regardless of the habitat of exposure. Interestingly, the specialist *Gyrodactylus sp.* burden showed only a habitat effect ($F_{2,629}=408.931$, $p<0.001$) being much more dominant in river.

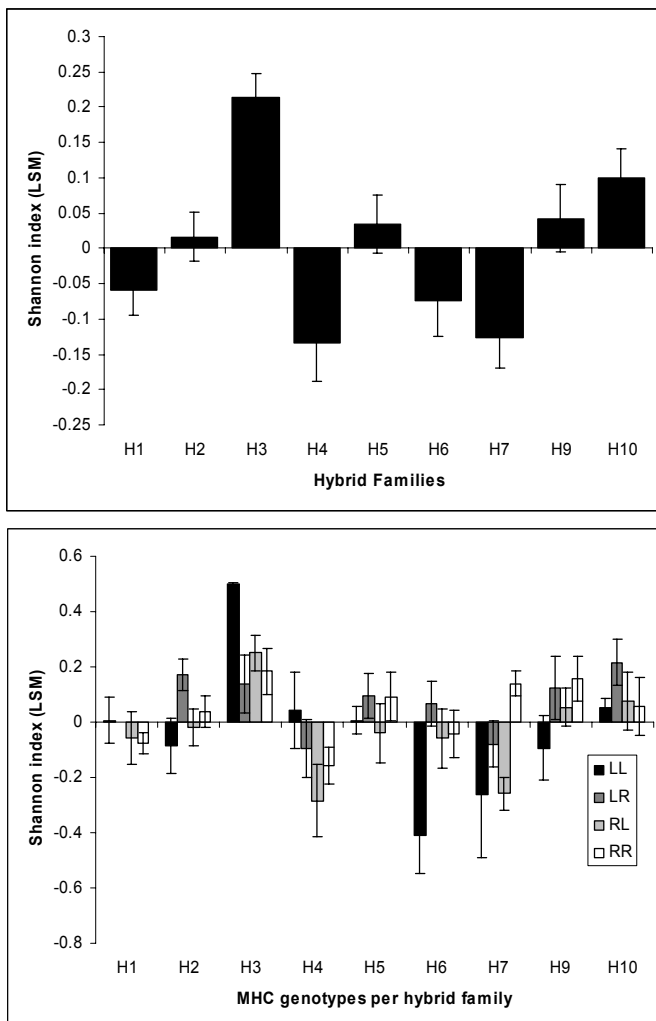
[Chapter VI Table 3 summarizes all the results for the different parasites investigated and the different hypotheses tested].

Immune assays. To obtain a parameter for granulocyte activation, we measured the oxidative burst activity in a chemoluminescence assay from the head kidney leucocytes (HKL). Sticklebacks exposed to the river showed higher zymosan-induced respiratory burst compared to fish exposed to the lake ($F_{1,629}=67.5173$, $p<0.001$). We could also observe an origin effect

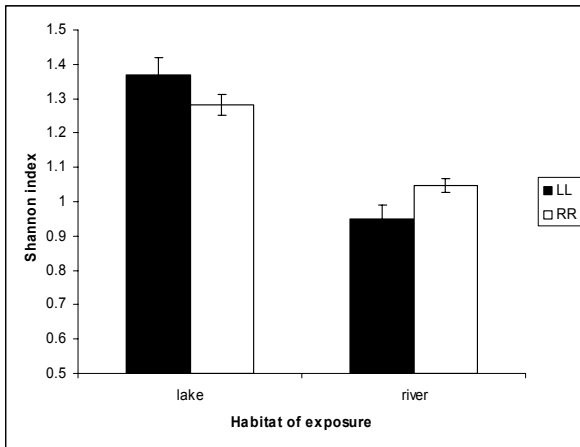
with pure lake lines displaying a higher respiratory burst than river fish ($F_{1,629}=16.703$, $p<0.001$. Fig.5). When focusing only on hybrid fish and their MHC genotype, we did not detect any relation between oxidative burst and MHC genotypes ($F_{1,263}=0.921$, $p=0.431$). In both cases, we found a strong inter-family variation ($p<0.001$)

Often, immune activation has been investigated on the basis of spleen weight. We checked whether the splenosomatic index (spleen weight relative to total weight) was related to habitat, fish origin and their interaction. We found only a significant interaction (Habitat: $F_{1,629}=0.091$, $p=0.763$; Origin: $F_{2,629}=0.335$, $p=0.716$; Habitat x Origin: $F_{2,629}=14.574$, $p<0.001$).

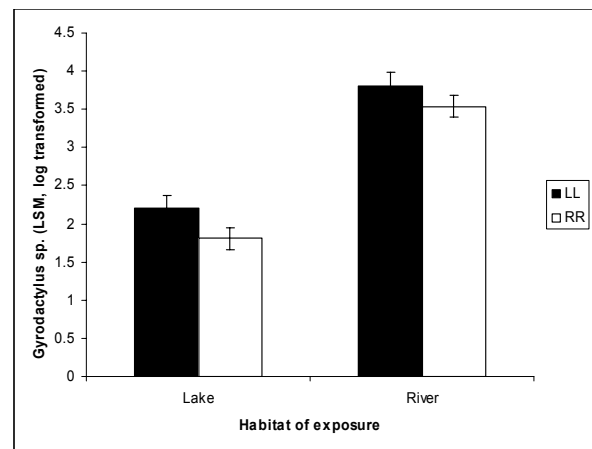
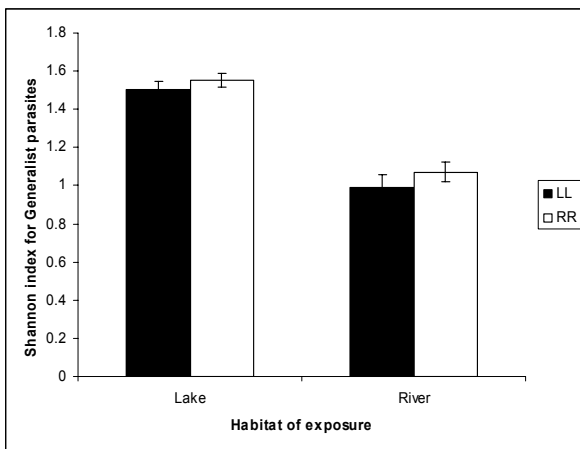
Growth rate. In order to estimate the general energy each fish needs to maintain its metabolism, we compared the fish weight (without gonads) between habitats, MHC genotypes and breeding origin. Generally, fish exposed to the river grew better than in the lake ($F_{1,629}=40.287$, $p<0.001$). Fish with pure river genome grew better in both habitats than pure lake and hybrid lines ($F_{2,629}=23.986$, $p<0.001$). We did not find any correlation between weight and MHC genotypes in the hybrid fish ($F_{3,263}=0.162$, $p=0.851$). Here as well, in both cases, we found a strong inter-family variation ($p<0.001$).



Chapter VI Fig. 2: Effect of family and MHC genotype on parasite load. The upper panel shows mean parasite load of stickleback in the nine hybrid families, exposed in the two habitats. The lower panel shows the parasite load for the 36 MHC genotypes in the nine hybrid families. Bars show means (+/- standard errors), computed from residuals of an ANOVA including habitat effect.



Chapter VI Fig. 3: Local adaptation of MHC origin. Parasite load of F2 hybrid stickleback carrying MHC genotype of different origin (LL vs. RR) and exposed to the natural parasite communities present in both habitat of exposure. a) for the Shannon index including all parasites, b) for the generalist parasites, c) for the specialist *Gyrodactylus sp.*. Bars show means (+/- standard errors).



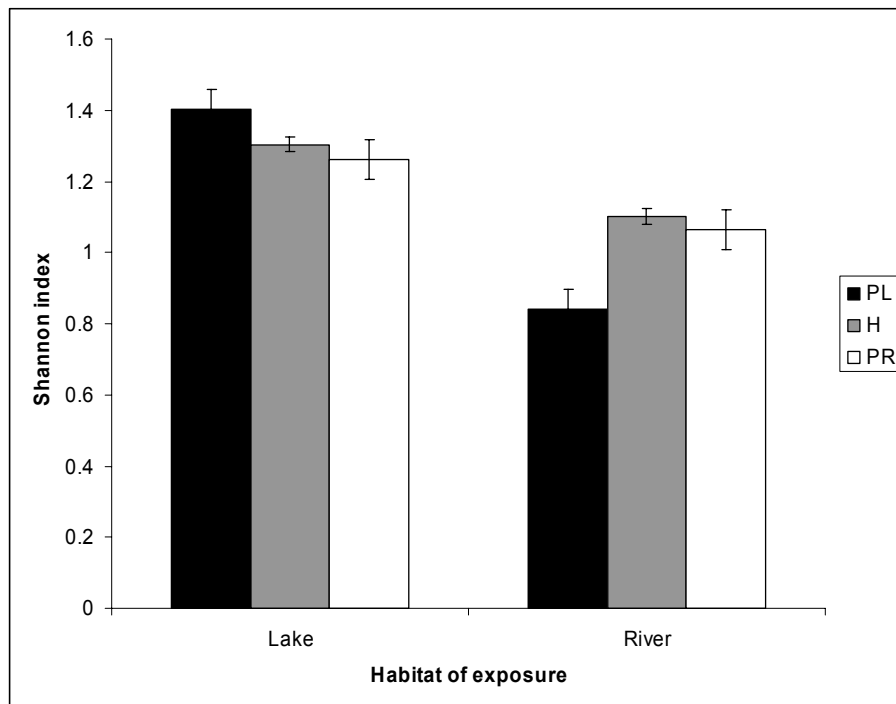
Discussion

It was recently proposed that MHC genes could act as magic traits by both adapting individuals to their local parasite fauna and leading assortative mating strategy to avoid disruption of adapted genotypes (Eizaguirre et al. 2008a). Here we tested for local adaptation of MHC genes, a first pre-requisite for MHC genes acting as a magic trait. Although interest for MHC genes has increased, experimental work testing evidence for MHC adaptation under natural environments is scarce (Rauch et al. 2006a; Consuegra & Garcia de Leaniz 2008). This might be due to effort of identifying the wide array of parasites a natural host population is confronted with (Thompson 1994) and to the difficulty of disentangling the role of MHC genotype from the background genome. In the present experiment we addressed the latter by using a two-lab generation breeding design, where we obtained replicated F2 fish that belonged to the same family but carried contrasting MHC genotypes. Exposing these fish to the natural habitat of their parents circumvented the limitation by the low number of parasites which can be handled in lab for experimental infections. Here we showed that in F2 hybrid fish, for which the background genome was randomized, the total parasite load was influenced by the MHC genotypes.

Chapter VI Table 3: Statistical details for three different parasite index: Shannon (total parasite load), Generalists (Shannon index obtained from only generalist parasites) and Specialist (log transformed number of *Gyrodactylus sp.*) a) test for influence of the different MHC genotypes in hybrids family, b) test for local adaptation of MHC genotypes, c) test for local adaptation of fish origin.

A) HYBRIDS	SHANNON		GENERALISTS		SPECIALITS	
Variables	F	P	F	P	F	P
MHC genotype	1.520	0.050	0.646	0.908	1.561	0.044
Family	6.048	<0.001	3.327	0.002	12.155	<0.001
b) Hybrids						
	Shannon		Generalists		Specialists	
Habitat	84.678	<0.001	182.678	<0.001	280.387	<0.001
MHC origin	1.629	0.183	0.958	0.413	4.815	0.003
Family	5.975	<0.001	3.479	<0.001	15.338	<0.001
Habitat x MHC origin	3.226	0.031	2.289	0.079	0.884	0.450
Family x MHC origin	1.548	0.210	0.551	0.936	1.137	0.314
Habitat x Family	1.262	0.141	0.324	0.957	10.999	<0.001
Habitat x Family x MHC genotype	0.926	0.540	0.8824	0.227	1.077	0.378
c) All fish						
	Shannon		Generalists		Specialists	
Habitat	213.862	<0.001	328.364	<0.001	408.931	<0.001
Origin	2.333	0.098	17.201	<0.001	1.372	0.254
Habitat x Origin	16.137	<0.001	0.831	0.436	0.718	0.488

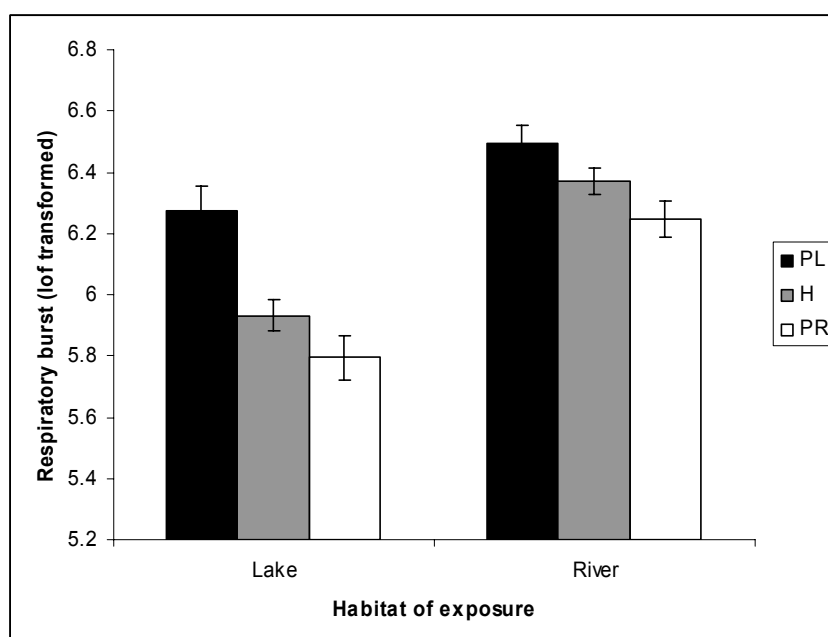
This clearly shows that MHC genotypes are not equal facing the parasites and that natural selection can play a crucial role in selecting best adapted MHC genotype. Although the effect was lower than the variation between families, we report the first field experimental evidence for a role of MHC genes in local adaptation to parasite resistance. The strength of the observed effect was in the expected range (Apanius et al. 1997). Our results contrast with a previous attempt of determining the role of MHC genes in parasite resistance where no effect was found (Rauch et al. 2006b). The discrepancy could stem from the longer exposure time in our study. Indeed, timing of exposure appears to be important for the pathways included in the adaptive immune system due to the production of antibodies (Janeway et al. 2001).



Chapter VI Fig. 4: Effect of fish origin and habitat of exposure on parasite load. Bars show mean Shannon index (+/- standard error)

While some innate immune mechanisms can show specificity in their response (Schmid-Hempel & Ebert 2003) the majority are, however, rather unspecific (Parham 2003). The adaptive immune system on the other hand is characterized by high specificity due to somatic rearrangement of antigen receptors. To mount an adaptive immune response takes up to two weeks in mammals and up to several weeks in ectotherms such as fish (Whyte *et al.* 1997). Interestingly splitting the parasite burden into specialist vs. generalist parasites revealed that the burden varied with MHC genotype only for a specialist parasite, the trematode *Gyrodactylus sp.* but not with generalist parasites.

A recent meta-analysis revealed a complex system where the local pattern of host-parasite co-evolution is a function of the parasite strategies (Lajeunesse & Forbes 2002). In brief, in a system driven by specialist parasites, these specialists should perform better on the local host compared to generalist parasites. Whereas in a system driven by generalist parasites, local hosts should perform better than migrants. Following this idea, we tested for MHC local adaptations in the hybrid lines. First, we noted that fish exposed to the lake displayed a higher total parasite load. This result was consistent with previous studies from populations inhabiting the same drainage system (Wegner *et al.* 2003b; Kalbe & Kurtz 2006; Rauch *et al.* 2006b; Scharsack *et al.* 2007).



Chapter VI Fig. 5: Effect of fish origin and habitat of exposure on respiratory burst. Bars show mean Shannon index (+/- standard error).

We found a pattern of local adaptation of parasites to local MHC genotypes, as fish with an MHC originating from lake (LL) were more infected in the lake habitat compared to fish bearing RR MHC genotypes. In turn, fish with an MHC originating from river (RR) were more infected in the river compared to fish with LL MHC genotype. Most surprisingly, this pattern was different when focusing only on generalist or specialist parasites respectively. Generalist parasite infection was not related to MHC genotype in any of the two habitats. In contrary, investigating parasite burden from a specialist parasite (*Gyrodactylus sp.*) revealed an advantage for fish bearing MHC genotypes originating from the river habitat (RR). This suggests that host MHC-parasite interaction is better seen with a specialist parasite. Generally, *Gyrodactylus* infection is higher in river than in lake (this study, Kalbe et al. 2002; Eizaguirre et al. Submitted a), what could explain the advantage for river MHC genotypes, which might have evolved to confer resistance to this specific, but dominating parasite. Although carried out on a lake population, a recent study (Eizaguirre et al. submitted b) showed an even tighter connection between the specialist *Gyrodactylus sp.* and a specific MHC haplotype, which might be the result of the high abundance of *Gyrodactylus* in that year. Fish carrying a specific haplotype were better at eliminating the parasite which in turn was reflected on the fish length. Fish length directly translated into Darwinian fitness, i.e. reproductive success. The results of the present study point in the same direction where host immune system (MHC) and specialist parasite are involved in a co-evolutionary arms race, where negative frequency dependent selection could play a crucial role (Doherty & Zinkernagel 1975; Takahata & Nei 1990).



Chapter VI Pic. 1: Pictures showing the habitats in which the cages were located. Right: picture of the slow flowing river, the Malenter Au. Left: picture of the lake Großer Plöner See.

The interaction observed on the total parasite load could arise from a habitat specific optimal trade-off of the host immunity between mounting a specific response against specialist parasites and activation of the innate immune system for fighting generalist invaders. This could arise from the non independence of both branches of the immune system (Dixon & Stet 2001).

This is best represented by the role of fish background genome (pure lake, pure river and hybrids) in the general local adaptation. Here we found as well that pure lake fish harbored a higher parasite load in lake than pure river fish, whilst pure river fish were more infected in river habitat, indicating that the local parasites adapted also to other components of the host immune system than MHC. Hybrid fish stand at an intermediate level of parasitisation. Again, total parasite load could reflect an optimal resistance strategy of the fish. Indeed, specialist parasite load was higher in river but was not related to fish genome origin, indicating the highly specific adaptation of the specialist parasites to only the host MHC and not to the rest of the genome. This was not the case for generalist parasites: Pure lake lines displayed a lower infection in both habitats, suggesting that the lake genome provides a variant of the immune system most adapted to cope with large numbers of generalist parasites. This is supported by the facts that most of the generalist parasites are extremely prevalent in lake and very little present in river and that the number of MHC alleles is generally higher in the lake, which could reflect a broader detection of pathogens. This observation is congruent with previous lab experiments for selection of a generalist parasite, the trematode *Diplostomum pseudospathaceum* (Wegner et al. 2007). In this study, sticklebacks originating from susceptible parents displayed higher parasite infection than fish originating from resistant lines. This resistance was linked to the innate immunity. However, highly susceptible lines compensated lower resistance by increasing MHC expression (see as well Wegner et al. 2006) bringing further evidence for the tight link between innate and acquired immune system. We could thus postulate a co-evolutionary process between host innate immune system and

generalist parasites potentially backed-up by MHC heterozygosity. Again supported by the fact that pure lake fish showed higher MHC heterozygosity. A certain level of adapted MHC heterozygosity was proposed to decrease parasite infection as shown in different surveys and laboratory experiments (e.g. Doherty & Zinkernagel 1975; Penn et al. 2002; Wegner et al. 2003a).

Further supporting such co-evolution between host innate immune system and parasites, we showed that fish of lake origin always displayed higher oxidative burst than river fish and this independent of the MHC genotype. The respiratory burst reaction, which is based on the production of reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2) or superoxide anions (O_2^-) by granulocytes and macrophages is an innate immune mechanism of major relevance. This is interpreted as an adaptation to the higher generalist parasite abundance in the lake, as shown experimentally in sticklebacks infected with the generalist *Diplostomom pseudospathaceum* (Kalbe & Kurtz 2006). However, this result contrasts with findings from Scharsack *et al.* (2007) where river sticklebacks displayed a higher oxidative burst than lake fish.

Another striking difference between sticklebacks from the different origins was the relative size of their spleen, which is an important organ of the immune system. Lake sticklebacks in lake had much higher spleen weights than fish with river genome, while river fish displayed a higher spleen weight in river. Due to the reciprocal exposure design of our experiment, we can conclude that the actual size of this lymphatic organ seems to follow the pattern of local adaptation, and cannot be regarded a general characteristic of these populations. It was recently proposed that lower spleen size in the three-spined stickleback reflected best MHC adapted immunity and provided fish with higher fitness (Kalbe et al. submitted). However, the mechanistic link awaits more experimental support.

In summary, we have conducted a field experiment where lab-bred fish were exposed for a substantial period of time to a range of natural parasites and could show that MHC genotype matters as does the genetic background. Interestingly we pointed out that MHC genes were mainly involved in interactions with specialist parasites, whereas genetic background and potentially MHC heterozygosity were important to resist generalist invasion. Thus, prerequisites of a co-evolution between hosts and parasites have been demonstrated and met the first assumption for MHC to be considered as magic trait.

We want to stress out that we are aware about the unfinished stage of this study and are confident to explain the observed processes in more detail with further analyses such as those

including MHC expression, effects of specific MHC haplotype and trophic specialization with stable isotope signatures.

Acknowledgments

We thank G. Augustin, D. Martens and H. Deiwick for technical assistance with the cages. We also thank S. Bartel, K. Brzezec, W. Derner, G.Schmiedeskamp, I. Schultz, K. Weiss for inestimable help with the dissections and lab assistance. In addition, we thank the county of Schleswig-Holstein and the city of Ploen for permitting us to run the outdoor experiments in the lake and the river.

Chapter VII

Parasites maintaining MHC polymorphism: an experimental test for negative-frequency dependent selection.

Study performed in collaboration between Christophe Eizaguirre, Tobias L. Lenz, Joern Scharsack, Martin Kalbe and Manfred Milinski.

Abstract

Genes of the Major Histocompatibility Complex are the most polymorphic genes in vertebrates and play a crucial pleiotropic function in immune defense and mate choice. Theoretical and observational work suggests that MHC polymorphism might be maintained by negative frequency-dependent selection by parasites. Due to a restricted optimal number of MHC loci, an individual cannot display the extreme MHC allelic diversity present in the population. Thus, individuals carrying specific MHC alleles that confer resistance against currently predominating parasites should perform better and should be favored during female mating decision. We designed an experiment to test for negative frequency-dependent selection not only including parasite pressure but also competition among males and mate choice. We created 6 replicate populations of three-spined sticklebacks, formed of a number of lab-bred families in equal proportions and equal sex ratio. Three of these populations were exposed to the nematode *Camallanus lacustris*, while the three others were confronted with the nematode *Anguillicoloides crassus*. These parasites are present in the original population from which the founders of the lab-bred populations were caught. Letting the system evolve in controlled outside tanks, we predicted that females would mate with partners possessing resistance alleles against the experimental parasite and thus the frequency of such alleles would increase in the following generation. After raising the collected eggs in the lab, we confronted the offspring with both parasites again. We hypothesized that fish from parents that faced one parasite, e.g. *Camallanus lacustris*, will be less heavily infected by this parasite than those coming from parents that encountered *Anguillicoloides crassus* and vice versa.

Introduction

Host-parasite co-evolutionary arms races (Van Valen 1973) could be sufficient to explain the maintenance of sexual reproduction - a fundamental question in evolutionary biology (Hamilton 1980; Hamilton et al. 1990). Indeed, theory predicts that sexual reproduction overcomes the disadvantage of the low evolutionary rate in asexual hosts, compared to their rapidly co-evolving parasites (Maynard Smith 1976). The impact parasites have on the evolution and ecology of their hosts depends on their virulence. Virulence is usually defined as the parasite-induced loss of fitness (Ebert & Hamilton 1996) and varies with host genotypes. The principal underlying assumption is that genetic variation for host-parasite interaction exists within populations and leads to differential fitness between co-evolving organisms. Such host-parasite co-evolution maintains polymorphism in parasite virulence and host resistance genes (Frank 2002).

Genes of the major histocompatibility complex (MHC) are an important part of the vertebrate adaptive immune system (Klein 1986; Janeway et al. 2001). MHC genes encode MHC molecules that transport antigens from the cytoplasm and present them at the cell surface to T-cells. The T-cells recognize pathogen-derived peptides and subsequently initiate a specific immune response. Particularly, the MHC class II genes are involved in the immune answer against extra-cellular parasites by encoding MHC molecules that present exogenously derived peptides to CD 4+ T helper cells (Klein 1986; Janeway et al. 2001).

MHC genes are the most polymorphic genes found in jawed vertebrates (Bodmer 1972; Apanius et al. 1997). The relative roles of different processes in the maintenance of polymorphism at MHC loci are still under investigation, but balancing selection is thought to play an important role. High intra- and inter-individual polymorphism is thought to be parasite-mediated and maintained by heterozygote advantage (Doherty & Zinkernagel 1975; Carrington et al. 1999; Suarez et al. 1999; McClelland et al. 2003) and/or by negative-frequency-dependent selection (Bodmer 1972; Takahata & Nei 1990; {for review see Apanius et al. 1997; Milinski 2006; Piertney & Oliver 2006). Theoretical works showed that heterozygote advantage alone cannot maintain MHC polymorphism (de Boer et al. 2004a; Ding & Goudet 2005). Moreover, theory would predict a stable polymorphism (Slade & McCallum 1992) that has so far not been reported. Under negative-frequency-dependent selection, a rare allele is more likely to be resistant to common parasite genotypes, and will therefore confer selective advantages to its bearer. Consequently, such an allele should increase in frequency in the next generation. However, parasite adaptation to host resistance should lead to selection for a parasite, or parasite strain, which is more virulent against this common genotype. In support of

this model, many associations between MHC alleles and resistance to parasitism have been found in natural populations (e.g. Hill et al. 1991; Langefors et al. 2001; Grimholt et al. 2003; Harf & Sommer 2005; e.g. Bonneaud et al. 2006b), (reviewed in Milinski 2006; Piertney & Oliver 2006). Vertebrates present a reduced optimal number of MHC loci and cannot display the entire potential MHC allele pool present in the populations (Lawlor et al. 1990; Janeway et al. 2001; Robinson et al. 2003). Because of this restriction, MHC alleles fluctuate over years. Although theoretically straightforward, to our knowledge, only a handful of studies provided hints of a potential negative-frequency-dependent scenario of resistance with MHC genes. Surveys of populations of great reed warblers (Westerdahl et al. 2004), sticklebacks (Wegner 2004) and soay sheep (Charbonnel & Pemberton 2005) represent the seldom examples of significant variation in MHC allele frequencies between successive age cohorts. Such variations were explained by potential fluctuating selective pressure from parasites. Contrary to the heterozygote advantage, negative-frequency dependent selection will maintain a dynamic polymorphism with fluctuating allele frequencies (Slade & McCallum 1992). Local parasite-driven selection favours some alleles and disfavors others in frequency-dependent selection, and so will female mate choice.

There are two different ways for an allele to increase in frequency: either selection against individuals lacking this resistance allele, or specific mate choice for this allele. There is growing evidence for MHC-based mate choice, e.g. in mice (Yamazaki et al. 1976; Potts et al. 1991; Penn & Potts 1998) in birds (Richardson et al. 2005; Bonneaud et al. 2006a), in fish (Reusch et al. 2001a; Aeschlimann et al. 2003; Milinski et al. 2005; Forsberg et al. 2007; Consuegra & Garcia de Leaniz 2008) and in primates (Schwensow et al. 2008). For instance, female sticklebacks can assess their potential partner's MHC make-up via perception of corresponding peptide ligands in odor signals (Milinski et al. 2005). Studies on great snipes and sticklebacks reported evidence for mate choice for males carrying specific MHC alleles (Ekblom et al. 2004; Eizaguirre et al. submitted b). Thus, MHC genes present the necessary pre-requisites to investigate negative-frequency-dependent selection.

Using the three-spined stickleback (*Gasterosteus aculeatus*) as model organism, we designed an experiment to test for negative frequency-dependent selection including both parasite pressure and female mate choice. We used large outside concrete tanks in which the fish populations could evolve without any uncontrolled parasite or predatory pressures. We created 6 replicate populations: each was formed from an equal mixture of 6 different lab-bred families (F1 generation). Three experimental populations were exposed to the nematode *Camallanus lacustris*, while the three others were confronted with the nematode *Anguillicoloides crassus*. Both parasites were common in the population where the parental generation was caught. We

assumed that females would mate with partners carrying alleles conferring resistance against the experimental parasite. This should lead to an increase in frequency of particular alleles in the following generation. During the reproductive period, we collected egg clutches and raised the offspring (F2 generation) under standardized conditions. After a certain growth period, we exposed the F2 generation to both parasites. We hypothesized that fish originating from parents that faced one parasite, e.g. *C. lacustris*, would be less heavily infected by this parasite than those coming from the population that encountered *A. crassus*; and vice versa. Investigating the association between parasites and specific MHC alleles, we aimed to demonstrate the role of negative-frequency-dependent selection in maintaining MHC polymorphism.

Materials and Methods

This section of the manuscript describes the different steps of the experiment that have been performed so far. The fish of the F2 generation have not yet been dissected and therefore results are not available. In order to remain blind with respect to the parasitisation, some molecular work has been performed but not analyzed.

Study organism. Three-spined sticklebacks were caught in the lake Großer Plöner See (54° 9'21.16"N, 10°25'50.14"E, Germany) in May 2006. After catching, fish were held singly in 16l tanks under summer conditions (day light 16/8h, 18°C air and water temperature). Males were provided with artificial nesting material (sand and nylon threads). We randomly crossed fish in order to obtain lab-bred sibships, however each individual was only used once. Breeding took place naturally, i.e. males and females were allowed to display natural courtship behavior and females spawned into the male's nest. Clutches were left for one hour in the nest after fertilization before being taken out. Individual egg clutches were incubated in aerated well water (with 0.04 ppm malachite green) at 18°C, until hatching. We obtained 10 clutches and randomly kept six for the experiment. The fish stayed under summer condition (18°C, 16/24 day light) until November. Then, the fry was transferred to autumn conditions for two weeks (12°C, 12/24 day light,) and to winter conditions for three weeks (6°C, 8/24 day light). Eventually the fish were brought to spring conditions (12°C, 12/24 day light,) for a 5 month period. Eleven days before release into the concrete enclosures (see below), the fish were accustomed to a longer day light and higher temperature to meet the external conditions (18°C, 16/24 day light).

DNA extraction, microsatellites and sex typing. In order to identify fish, a piece of the second dorsal spine was clipped for DNA typing. DNA extractions were performed using

DNAeasy purification kit (Qiagen, UK,) following the manufacturer's protocol. All the fish were typed for 9 microsatellites combined in 2 different PCR multiplex protocols (Kalbe et al. submitted). Moreover, fish were typed for sex using Griffiths *et al.*'s (2000) protocol adapted for high-throughput typing: One PCR primer was fluorescently labeled and the signal was subsequently detected on an automated sequencer. For this, 2 μ l PCR product were mixed in 8 μ l HiDi before a 2 minute denaturation step (90°C) followed by snap-cooling (4°C) for 5 min. An Applied Biosystem 3730 DNA Analyzer was used and runs were analyzed with GeneMarker (v.1.70). In the chromatograms males were represented by two peaks (370bp and 600bp) while females displayed only one peak (600bp). During DNA extraction and sex typing, fish were kept singly. Length and weight were recorded.

Laboratory populations and concrete outside tanks. By randomly assigning 7 males and 7 females per family to each of the six concrete outside tanks, we created six populations of 84 fish each and with an equal sex ratio. The outside concrete tanks had a capacity of about 46m³ (4.8m x 4.8 m x 2m). On one side, the tanks had an additional shallower part (1m width x 0.5 m depth) in order to provide male sticklebacks with nesting areas. In each tank, the shallow area was divided into six territories of 80x60 cm using bricks (see figure 1). Nesting material and sand were added at the beginning of the experiment.

Fish Infection

- First generation (F1)

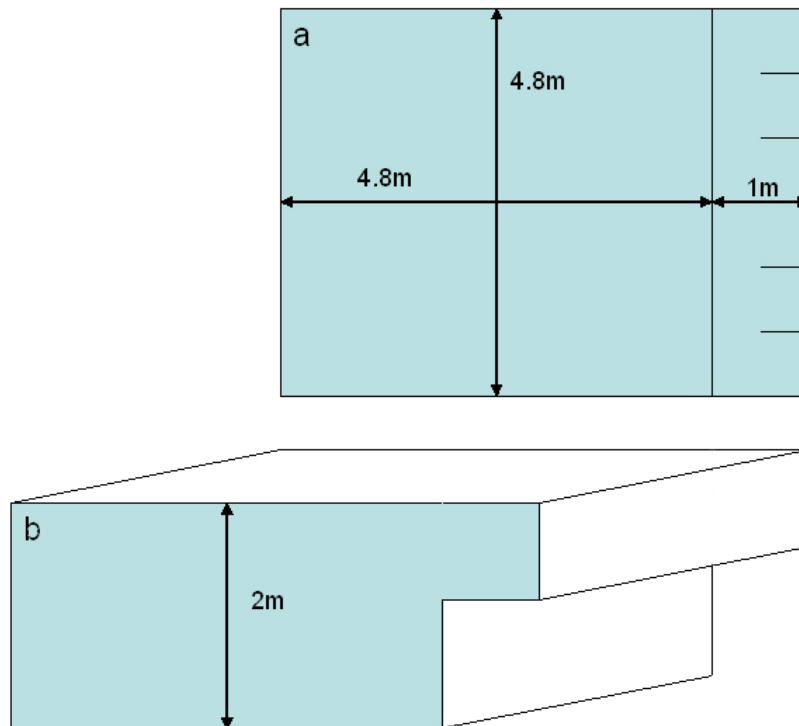
Before release into the outside tanks, the 604 sticklebacks from the F1 generation were infected twice with one of two different parasites. Three lab-populations were exposed to the nematode *Camallanus lacustris*, while the three others were exposed to the nematode *Anguillicoloides crassus*. These two parasites were chosen because they occur naturally in the population where the parental generation originated from and because infection has been shown experimentally to be MHC-dependent (Wegner et al. 2003a).

C. lacustris ripe females were obtained from the blind sacks of perch guts. Larvae were isolated from single worms and counted. *A. crassus* larvae were collected after maternal worms, isolated from eel swim-bladders, had released eggs. These two parasites necessitate different intermediate hosts to achieve their complete life cycle, one of which is the three-spined stickleback. Stickleback infection occurs through trophic transmission. In the lab, we used *Macrocyclops sp.* copepods as intermediate hosts (see as well Wegner et al. 2003a; Krobbach et al. 2007). In the first round of fish infection, 6.000 copepods were exposed to more than 48.000 *C. lacustris* larvae. This resulted in an average of 8 larvae/copepod. We also exposed 3.000 copepods to 7.600 *A. crassus* larvae resulting in an average of 2.5 larvae/copepods. The infected copepods were kept during three weeks in 1 liter tanks in

groups of 100 before fish infection. They were fed twice a week once with artemia and once with paramecia. This protocol was followed for each copepod infection.

To stimulate the adaptive immune system, a second round of infection took place 6 weeks later. We exposed 6.000 copepods to more than 48.000 *A. crassus* larvae and 3.000 copepods exposed to 21.000 *C. lacustris* larvae.

The infection status of the copepods was determined by microscopically counting nematode larvae inside the copepod before exposure to the fish. Variation in the intensity of infection in copepods prevented the exposure of all fish with an identical infection dosage. For the two rounds of infection, fish were kept singly in tank overnight and only fed with the infected copepods.



Chapter VII Fig. 1: Schematic representation of the concrete outside tanks. a) shows a view from above the basins, while b) shows a perspective view with the breeding area.

- **Second generation (F2)**

Fry was raised in the lab. After 8 weeks, we isolated the fish in group of 20 in order to control for density effect in the growth. We randomly picked 30 clutches. Half originated from the outside tanks where the F1 fish had been exposed to *C. lacustris* and the other half from the outside tanks where the F1 fish had been exposed to *A. crassus*.

For each clutch, 15 randomly assigned F2 fish were exposed to *C. lacustris*, while another 15 were exposed to *A. crassus*. This resulted in a total of 900 fish to be infected, each half with one of the two parasites.

Here as well, in order to trigger the adaptive immune system of the fish, we exposed the fish twice to the parasites. For each exposure period, more than 7.000 copepods were bred in the lab and more than 40.000 parasite larvae were collected in the same way as for the F1 generation. Parasite larvae were always freshly collected from either perch guts or eel swim-bladders. Each copepod was checked individually and copepods were excluded if the exact number of larvae could not be determined.

In the first round, fish exposed to *C. lacustris* received 7 larvae, while the fish exposed to *A. crassus* received 9 larvae. In the second round of infection for the *C. lacustris* treatment, the fish were exposed to 7 parasite larvae, whilst the fish exposed to *A. crassus* received randomly from 6 to 9 larvae, because of limited availability of larvae.

Before exposure to the parasites, F2 fish were weighed and length was recorded. At the same time the second dorsal spine was clipped for further molecular analysis.

Egg collection. To estimate reproductive success and to infer mate choice, once a week, all stickleback nests from the outside tanks were detected by careful observations. All egg batches were collected and the nests carefully replaced in their original location. Egg batches were brought to the laboratory. Within a nest, clutches were separated on the basis of the eggs' developmental stages. Until hatching, eggs were kept using 1l glass jars filled with well water and provided with air supply (for eggs handling, see Kalbe et al. submitted).

F1 fish dissection. After 4 weeks in the outside tanks, the experiment was stopped since the number of collected clutches decreased markedly. Fish were recaptured and dissected. Dissection took place to record parasite intensity in the F1 fish. Dissections were done blind, so fish were checked for both parasites. Fish length, weight and inner organs weight were recorded. A piece of tail fin was cut for further molecular analysis.

Immunological assays. For immunological assays, leucocytes were isolated from head kidney (HKL). Head kidney preparations followed Scharsack *et al.*'s protocol (2004). Total cell numbers in HKL isolates were determined with the standard cell dilution assay according to

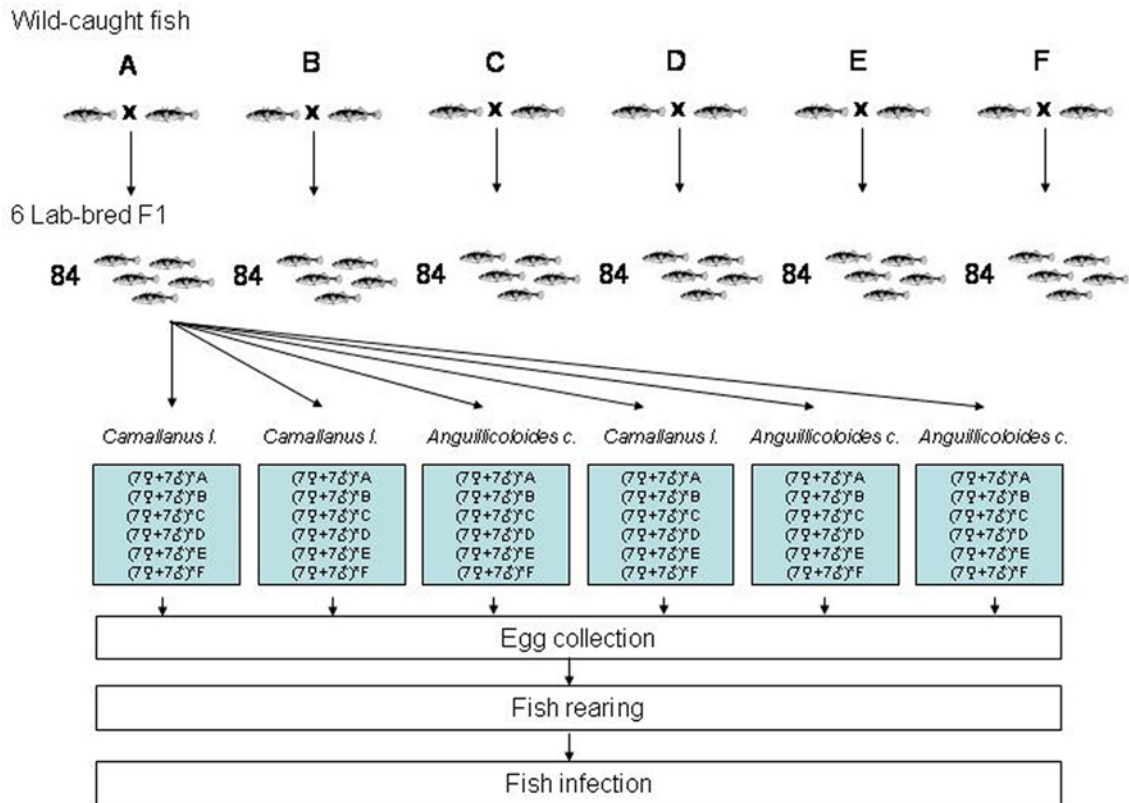
Scharsack *et al.* (2007). Additionally, flow cytometric measurements of freshly isolated HKL were used to determine proportions of granulocytes (FSC/SSChigh) and lymphocytes (FSC/SSClow) in individual HKL samples.

As important factor of the cell mediated innate immunity, the respiratory burst activity of HKL was quantified (Kurtz *et al.* 2004). As a parameter for activation of the adaptive immune system, frequencies of lymphocytes in G0-1, S and G2-M phases were acquired by DNA content analysis of red fluorescence intensity (propidium iodide labelling) of single cells from the lymphocyte gate (Scharsack *et al.* 2007) .

DNA extraction and microsatellite typing after dissection. DNA extractions from tail fin tissue were performed using the DNAeasy purification kit (Qiagen, Sussex, UK) following the manufacturer's protocol. All F1 fish were re-typed with the same 9 microsatellites as before the experiment plus an additional 6 to allow parenthood resolution. All were combined in 4 different PCR multiplex protocols (Kalbe *et al.* submitted). These markers were used to reliably identify the fish at the end of the experiment and to perform parenthood analysis on the collected clutches.

Cloning and MHC typing. The twelve wild-caught parental fish were cloned and sequenced. In order to identify all MHC class II*B* alleles of the exon 2, we cloned and sequenced a fragment running from Exon 1 to Exon 2 of the MHC class II*B* genes, employing cautionary PCR conditions according to Lenz & Becker (2008). Setting a primer in the highly conserved Exon 1 reduces the risk of potential primer bias during amplification. For each fish, 48 clones from at least two independent reactions were sequenced.

The MHC class II*B* diversity of the F1 and F2 fish was determined by reference strand conformation analysis (RSCA) according to Lenz *et al.* (in prep). We amplified the exon 2 of MHC class II*B* genes that encodes for the peptide-binding groove of the MHC II molecule. This exon has previously been suggested to be under parasite mediated positive selection as revealed by a high ratio of nonsynonymous over synonymous substitutions (d_N/d_S) (e.g. Reusch *et al.* 2004)



Chapter VII Fig. 2: Representation of the experimental design used for breeding and parasite exposure during the experiment.

Initial results

MHC diversity in wild-caught parental fish. Individual MHC diversity varied from 3 to 5 alleles. In total the 12 founder fish displayed 17 different alleles.

Egg collection. During the four weeks the F1 fish spent in the outside tanks, we collected 46 clutches. The total number of eggs per clutch varied from 29 to 529.

Parental parasite load. In the parental generation we observed a strong variance in parasite infection between parasites and between individuals. 78.2 % of the fish exposed to *C. lacustris* were infected while 42.1 % of the fish were infected with *A. crassus*. Infection with *C. lacustris* ranged from 1 to 28 with a mean infection of 8.7 worms. Fish exposed to *A. crassus* displayed a lower mean infection of 1.8 worm. The ranged of worm found in the swim bladder varied between 1 and 6.

Such variation encourages us to continue in our quest since differential host resistance represents the necessary pre-requisite for to negative-frequency dependent selection by parasites.

Discussion

Here we aimed to test the role of negative-frequency-dependent selection in the maintenance of MHC polymorphism.

If our hypotheses would be correct, female mate choice should lead to an increase in the frequency of resistance MHC alleles. For females, to be able to choose a mate with resistance alleles, males should signal their performance against parasites. Secondary sexual coloration of the throat of male stickleback has been investigated in length. Individual redness has been shown to indicate parasitization (Milinski & Bakker 1990a), body condition (Bakker & Mundwiler 1994) and MHC make-up (Jäger et al. 2007). Therefore, we expect males that are most able to resist parasitization to be best able to gain a territory, maintain a nest and attract females. During the experiment, we performed immune assays and can thus assess the potential trade-off between parasitization and activation of the immune system (Sheldon & Verhulst 1996). Fish with and without a specific resistance allele might use different components of the immune system to maximize resistance while minimizing energy allocated to the immune system.

Numerous hints exist in the literature to hypothesize the action of such parasite mediated negative-frequency dependent selection. First, various associations between MHC alleles and resistance to pathogen have been reported (e.g. Briles et al. 1977; Hill et al. 1991; Haldane 1992; Langefors et al. 2001; Lohm et al. 2002); for review see (Sommer 2005a; Milinski 2006; Pieltney & Oliver 2006). These associations are a pre-requisite to negative-frequency selection. Second, over time, the patterns of polymorphism maintained by heterozygote advantage and frequency dependence should be very different. The latter would be reflected by a strong variance in allele frequencies between generations, while heterozygote advantage predicts an even stable distribution of alleles (Slade & McCallum 1992). This last decade, MHC genes have been the focus of very diverse studies. Despite this diversity, all studies found a common trait: a strong variation of allele frequencies (e.g. Boyce 1996; e.g. Wegner et al. 2003b; Harf & Sommer 2005; Meyer-Lucht & Sommer 2005; Cutrera et al. 2006). Eventually, three studies, dealing with a bird (Westerdahl et al. 2004), a fish (Wegner 2004) and a mammal (Charbonnel & Pemberton 2005) as model organisms, revealed a dynamic fluctuation of MHC alleles between age cohorts; another pre-requisite to polymorphism maintained by frequency dependence (Slade & McCallum 1992).

Association between MHC alleles and resistance to parasite infection, dynamic fluctuations of MHC alleles as well as MHC-based mate choice are suggestive of negative-frequency dependent selection by parasites maintaining MHC polymorphism and ultimately explaining the necessary two-fold advantage of sexually reproducing over clonally reproducing organisms. However, negative-frequency dependent selection can become more complex. Competition of co-infecting parasites might lead to the paradox of susceptible MHC alleles.

Almost as frequently as resistance alleles, studies have reported associations between MHC alleles and infection disease susceptibility (Carrington et al. 1999; Hendel et al. 1999; Bonneaud et al. 2006b; Loiseau et al. 2008). The persistence of susceptible alleles represents an evolutionary paradox as there may be clear costs associated with carrying these alleles. Theoretically, alleles that have antagonistic effects on fitness can be maintained in populations (Zhang et al. 2002; Loiseau et al. 2008) because a susceptible allele towards a parasite may confer resistance against another. This would correspond to two simultaneous arms races between one host and two parasites, where parasite virulence between parasites would drive the system. However the prediction of antagonistic pleiotropy involving two parasites awaits empirical evidence.

Alternatively to a direct effect of antagonistic (resistance/susceptibility) MHC alleles on parasite infection, within-host-competition between parasites or parasite strains could result in the same pattern of susceptible alleles. Parasites exploiting the same resources in the host are potentially involved in competitive interactions and maintain each other in equilibrium. Specific immune recognition of different parasite antigens through MHC molecules can alter the competitive interaction between parasites and break the equilibrium. For instance, if allele A confers resistance against parasite P1, and if P1 and P2 are two parasites exploiting the same host, the allele A could reduce the competitive interactions between the two parasites. Theoretically this could result in an observed increase in susceptibility of P2 (Raberg et al. 2006; Loiseau et al. 2008). Although proposed, this theory as well needs experimental support that could be brought by further investigations based on our original experiment set-up.

The multitude of hints in favor of negative-frequency dependent selection by parasites deserved consideration and was the motivation to perform our experiment. We hope to better understand MHC polymorphism and thus contribute to the knowledge about one of the ultimate questions in evolutionary biology, namely the maintenance of sexual reproduction.

Acknowledgments

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Conclusion

In the main introduction I detailed the particular and important role of parasites in evolution. My doctoral research has aimed to find an answer to the question of how host/parasite interactions can drive evolution within species, between sexes and between species.

Given that most species on earth are parasites (Windsor 1998; Kuris et al. 2008), understanding the pressure they place on their hosts is a major challenge in biology. The tremendous variation of MHC genes exhibited by vertebrates can obviously not be neutral in face of natural selection and parasites are probably the source of this extreme polymorphism (Clarke & Kirby 1966; Klein 1986). It was proposed that negative-frequency dependent selection could be a mechanism maintaining MHC polymorphism (see for review Slade & McCallum 1992; see for review Milinski 2006) and we attempted to test this hypothesis (Chapter VI). Contrary to the latter, several studies have demonstrated the contribution of overdominance by showing that heterozygotes are able to detect a wider range of pathogens (Doherty & Zinkernagel 1975) and are therefore more resistant to infection (Carrington et al. 1999; Arkush et al. 2002; Grimholt et al. 2003). Nevertheless, not all studies found support for this hypothesis (e.g. Wegner et al. 2003a; Wegner et al. 2003b; e.g. Ilmonen et al. 2007). The latter studies used the stickleback as model organism.

The three-spined stickleback has been used as leading model organism to study host immune genes-parasite interactions. In this teleost fish, intermediate levels of MHC diversity were shown to be associated with lowest parasite burden (Wegner et al. 2003a; Wegner et al. 2003b). Since then, various studies have reported advantages to individuals with intermediate MHC diversity in several taxa (e.g. in birds (Buchholz et al. 2004; Bonneaud et al. 2006b), in reptiles (Madsen & Ujvari 2006). In sticklebacks, intermediate MHC make-up was also demonstrated to be associated with higher survival under stressful abiotic conditions (Wegner et al. 2008).

In addition, MHC genes have been shown to play a crucial role in reproductive strategies: for instance, female sticklebacks assess potential mates by incorporating multiple cues. In olfactory mate choice experiments, females preferred males sharing an intermediate MHC diversity (Reusch et al. 2001a; Aeschlimann et al. 2003). This assessment is performed through perception of correspond MHC peptides in odor signals (Milinski et al. 2005). Although cues are likely to be olfactory alternative cues may exist. Indeed, my colleagues and I have demonstrated an expected optimal MHC class II B allele diversity in males capable of maintaining a high quality nest. The nest could be considered as honest visual cue signaling

current condition. Such results indicate that females may react to a broad range of signals all, more or less dependent on MHC genes.

To test for female mate choice under semi-natural condition we have used field experiments (Chapter II & III). We have demonstrated that both laboratory-bred and wild-caught females based their mating decision on MHC genes and MHC-dependent traits. Studies on wild-caught fish shown that females could achieve a two fold advantage by mating assortatively: i) by choosing males sharing an intermediate MHC diversity, they optimize parasite resistance/ T cell depletion while ii) by choosing males with a specific MHC haplotype they insure high parental care and maximize egg protection and hatching. Although, mate choice oriented towards a specific MHC haplotype had previously been demonstrated in lekking great snipe (Ekblom et al. 2004), no previous studies have reported a direct link between Darwinian fitness and the role of parasites.

In this thesis, my colleagues and I have opened a new window concerning studies of MHC genes. We have proposed and demonstrated that MHC play a pleiotropic role in parasite resistance and mate choice (Chapter IV, V, VI). All over the northern hemisphere, the repeated successful colonization of habitats with subsequent occurrence of large natural populations means that sticklebacks represent an ideal candidate to investigate ecological process leading to speciation. Different arms races between hosts and parasites could be a major force driving population divergence and MHC genes could play a crucial role in this process.

To conclude, the challenge of this thesis was to cover many aspects from the angle of interactions between a host single gene complex and its parasites. We have revealed that MHC genes are correlated to visual and olfactory cues taken into account by females for mating to increase survival and reproduction likelihood of their progeny facing parasites. We have shown that MHC genes are involved in the process of speciation and that they could be regarded as “magic traits”. Future studies should focus on the generality of the mechanism proposed here. In addition, the products of genes are still poorly understood and most probably include significant insights to better understand the endless arms races between hosts and parasites.

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Glossary

Alleles:

An allele is an alternative form of a gene (one member of a pair in diploid organisms) that is located at a specific position on a specific chromosome. Usually alleles are coding sequences but this term is as well used for non-coding sequences (e.g. microsatellites).

Balancing selection:

Balancing selection refers to a form of selection that maintains genetic polymorphism within a population. It is thought that balancing selection is operated mainly by overdominance and negative frequency-dependent selection.

Ecotype:

Within a species, an ecotype is a genetically distinct population (or group of populations) that is adapted to its local environment.

Haplotype:

The term haplotype is a contraction of the term "*haploid genotype*". A haplotype is a combination of alleles at multiple loci that are transmitted together to the progeny.

Heterozygote:

A nucleus, cell or organism is said heterozygote if its DNA carries different alleles of the gene in question.

Homozygote:

A nucleus, cell or organism is said homozygote if its DNA carries only one unique allele of the gene in question.

Individual MHC diversity:

The number of different MHC variants within an individual. Due to duplications of MHC loci, more than two variants can be found in an organism. For simplicity they are called alleles here, although they might not stem from homologous loci.

MHC:

The major histocompatibility complex is a large genomic region found in most vertebrates. It is the most gene-dense region in the mammalian genome. It plays an important role in the

activation of the immune system, in auto-immunity and in mating strategies. Gene products of class I and class II present bound peptides to T-cells.

Microsatellites:

Microsatellites are short sequences of di- to tetra-nucleotide repeats of very variable length. Usually these sequences are not coding, supposedly neutral and can be extremely variable. The use of these extremely common sequences has opened a wide array of applications in genetic mapping, population genetics, paternity assignment etc.

Mutation:

Mutations are changes in DNA sequence of an organism.

Negative frequency-dependent selection:

Also called rare allele advantage, negative frequency-dependent selection refers to the fitness contributed by an alleles that is negatively correlated to its frequency in the population.

Overdominance:

Overdominance exists when heterozygous individuals have a fitness advantage over both homozygotes.

Parasites:

A parasite is an organism that lives in, or on another living organism, obtaining from it part or all of its organic nutrients, and causing damage to its host but which does not intend to kill its host.

Red queen hypothesis:

The rapid dynamics of antagonistic co-evolution are pictured by this metaphor from Lewis Carroll's book "Through the looking glass".

Speciation:

Speciation is the evolutionary process by which a new species arise.

Curriculum Vitae

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Education

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Lycée René Cassin - Bayonne (64), France.
- 2000-2002: DEUG of General Biology, option Earth and Universe Science.
University of Science Bordeaux I – Bordeaux (33), France.
- 2002-2003: Licence of General Biology (Bsc. General Biology), option General Biology.
University of Science Angers - Angers (49), France.
- 2003-2004: Master (first year) in “Biology of Populations and Ecosystems”,
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Paul Sabatier University -Toulouse (31), France.
Thesis: “Birds from Pacific Islands: theories and conservation”.
- 2004-2005: Master of Science “Ecology, Biodiversity and Evolution”.
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Publications

- **Eizaguirre, Christophe**, Laloï, David, Massot Manuel, Richard Muriel, Federici Pierre, and Clobert Jean (2007) Condition dependence of reproductive strategy and the benefits of polyandry in a viviparous lizard. *Proceedings of the Royal Society B: Biological Sciences* 274: 425-430.
- Jaeger Ilonka, **Eizaguirre Christophe**, Griffiths Sian.W., Kalbe Martin, Krobbach C.Kerstin, Reusch Thorsten .B. H., Schaschl Helmut & Milinski Manfred (2007) Individual MHC class I and MHC class IIB diversities are associated with male and female reproductive traits in the three-spined stickleback. *Journal of Evolutionary Biology* 20 (5): 2005-2015.
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Appendix I

RSCA genotyping of MHC for high-throughput evolutionary studies in the model organism three-spined stickleback

Tobias L. Lenz, Christophe Eizaguirre, Thorsten B. H. Reusch, Sven Becker

Abstract

Background: The highly polymorphic genes of the major histocompatibility complex (MHC) code for antigen presenting molecules of the adaptive immune system in all jawed vertebrates. The polymorphism is composed of a high total number of alleles as well as high nucleotide diversity on sequence level between any two alleles. It provides resistance against the natural parasite fauna and enables adaptation to new pathogen threads as well as co-evolution with sympatric parasites. Analysing this diversity and understanding the underlying mechanisms that maintain it require a reliable genotyping technique, optimised for such highly variable genes.

Results: We present a genotyping protocol using Reference Strand-mediated Conformation Analysis (RSCA), optimised for the duplicated MHC class IIB genes of the Three-spined stickleback. In addition we used a comprehensive plasmid library of MHC class IIB alleles to determine the nucleotide sequence of RSCA allele peaks. Verification of the RSCA typing by cloning and sequencing shows high congruency between both methods and provides new insight into the polymorphism of the stickleback MHC. Analysis of the single plasmid library additionally reveals the high resolution and reproducibility of this technique.

Conclusion: This new RSCA genotyping protocol offers a less laborious, but equally sensitive and reliable way to determine the MHC allele repertoire of Three-spined sticklebacks as cloning. It therefore provides a valuable tool to employ this highly polymorphic and adaptive marker in future studies of an emerging model system for host-parasite co-evolution and ecological speciation.

Background

The major histocompatibility complex (MHC) is one of the most polymorphic regions in the vertebrate genome [1, 2]. It consists of genes, which code for cell surface molecules that present self and non-self antigens to T-cells and therefore play an essential role for the recognition of pathogens invading the body [3]. The high polymorphism in this system, which is usually composed of a high total number of alleles as well as exceptional nucleotide diversity on sequence level between any two alleles, is thought to enable populations to fight their natural, but co-evolving parasite fauna [4-7].

The MHC has intrigued scientist around the world, because of its crucial functions and is increasingly being studied in natural populations. Yet, its polymorphism also presents a serious challenge to the scientific community as reliable genotyping methods for such variable and often duplicated loci are scarce and difficult to apply and optimise. In many mammalian species it is nowadays possible to target single loci, because of relatively stable structural organisation and gene orthology within the mammalian MHC [8, 9]. In contrast, non-mammalian species show a substantially lower conservation in their MHC structure [8]. This leaves a large black box concerning the organisation of the MHC and gene orthology in most less investigated species and locus specific typing is hardly possible [10-15]. Therefore it is of major concern for our understanding of the MHC and its role in evolution and ecology, to find a reliable typing method for MHC diversity that is not depending on detailed knowledge about the respective species' organisation of the MHC and is able to cope with larger numbers of alleles. The development of MHC primers for new species can be achieved by starting from sequence information of closely related species, because of certain sequence conservation of parts of the MHC genes [10, 16]. This approach facilitates the amplification of MHC genes in new species without excessive genome screens, but potentially risks lower specificity of the primers. Additionally, more and more species under investigation show signs of very recent gene duplications in the MHC. Both, conserved sequence motifs as well as sequence similarity due to recent duplication often lead to co-amplification of several loci and a large number of alleles with undistinguishable locus origin that have to be separated simultaneously [e.g. 12, 13, 14, 17, 18].

The most reliable and trusted method for allele identification so far is sequencing with prior allele separation via cloning of PCR products. However, this is costly and requires a lot of effort, which increases exponentially with the expected number of alleles. As picked clones represent only a small sub sample of the amplified fragments, this method is more prone to overestimating PCR artefacts, especially if only a small number of clones of each individual is being sequenced to save resources [19, 20].

Faster and less expensive methods for genotyping of unknown alleles have been employed, the most common ones being DGGE [Denaturant gradient gel electrophoresis, 21] and SSCP

analysis [Single-strand conformation polymorphism, 22]. DGGE separates different double-stranded sequence variants according to their denaturation characteristics and provides a single band per variant. SSCP analysis achieves separation of variants due to mobility differences of the two complementary single strands in a non-denaturing matrix and therefore provides two bands per allele [Both methods discussed in 23]. SSCP can also be run in a capillary system, using automated laser detection of labelled single strands and by that producing peaks instead of bands [CE-SSCP, 24]. Factors that complicate allele identification with these methods, which produce only one and two characteristic values per allele respectively, are large numbers of distinct variants in the pool, which limit the resolution through overlapping peaks, and run to run variation in the gel matrix, which confounds the comparison of different cohorts of individuals that have been typed at different time points.

A disadvantage of indirect typing methods is as well the lack of sequence information. The MHC genes comprise certain amino acid residues that are actively involved in antigen binding and presentation while most of the others residues have more conformational importance [25]. The former residues usually stand under strong balancing selection, shown by a high rate of non-synonymous substitutions between alleles, while the latter are less variable. With increasing insight into the evolutionarily important mechanisms of antigen binding it becomes a key aspect to resolve alleles to the sequence level, enabling sequence and even residue-specific analysis of adaptation [26-29]. While this lack of sequence information in SSCP and DGGE can be overcome by running the fragments on a physical gel and subsequently sequence the detected bands, this is not possible for high throughput typing protocols like CE-SSCP.

Reference Strand-mediated Conformation Analysis [RSCA, 30] is a relatively inlaborious genotyping technique, which experiences increased popularity. Here, all sequence variants are amplified simultaneously and hybridised to a given fluorescent labelled reference strand, forming a heteroduplex with distinct mismatches for each variant. Together with the homoduplex of the reference strand, these heteroduplexes are then separated according to their specific mobility in a given non-denaturing environment, which depends on the distinct tertiary structure of each heteroduplex. This mechanism provides a significant advantage over other commonly used indirect typing techniques, because it produces as many mobility values per allele as desired by using several distinct reference strands and therefore creates a multi-dimensional coordinate for each allele. This enables differentiation of highly similar sequence variants [31].

Since its development by Argüello *et al.* [30], RSCA has been used in a number of species to type MHC loci, namely in humans [e.g. 32], other primates [e.g. 33, 34-36] and in several non-primate mammalian species [e.g. 37, 38-41]. However, its application in non-mammalian

species is still rare. To our knowledge the only studies so far have been performed in Brown trout [42] and Red jungle fowl [43], where only one and two loci have been addressed at a time respectively.

Three-spined stickleback is an emerging model species that is increasingly being used to study evolutionary phenomena like, for instance, sexual reproduction [e.g. 15, 44-47], host-parasite co-evolution [e.g. 48, 49], ecological speciation [e.g. 27, 50, 51-55] or evo-devo [e.g. 56, 57, 58].

We focussed on the class II genes of the stickleback MHC, as they were shown earlier to have significant influence on parasite resistance [48, 59], mate choice [15, 44, 47], survival [60], lifetime reproductive success (Kalbe, submitted; Eizaguirre, submitted) and potentially even speciation (Eizaguirre, submitted).

Previously, MHC class IIB genes of the Three-spined stickleback have been analysed using CE-SSCP [11, 15]. A crucial point in genotyping is the number of different loci that will have to be separated. In the past, the number of MHC class IIB loci in the stickleback had been reported to be as high as 6 [61], but this was recently corrected to 2-4 [62]. Potentially due to recent origin of locus duplication or ongoing inter-locus recombination [63], it is not possible to address these loci singly and therefore several alleles per individual have to be differentiated, independent of the genotyping technique used. This poses a strong challenge even to cloning, the gold standard genotyping method, due to the increased rate of sequence artefacts [20]. The established CE-SSCP protocol for the stickleback was already known to provide limited resolution by not separating all sequence variants [15] and finally reached its limits when following a comprehensive breeding design with fish from two different populations, due to which a new set of eight primer combinations had to be developed [64]. This increased the number of necessary PCR reactions and SSCP runs per fish substantially and inhibited high-throughput genotyping, the initial reason for using indirect typing methods.

We therefore developed a new genotyping protocol employing RSCA and a comprehensive plasmid library of MHC class IIB alleles to determine the nucleotide sequence of RSCA allele peaks. Results of the allele and sequence specific RSCA typing were verified using the gold standard technique of cloning and sequencing.

Methods

- Primer development

Primer design in the MHC region is a special challenge. If primers within the most variable exon2 are needed, they should on the one hand be as locus specific as possible, but on the other hand amplify all alleles that could be found on this locus. In the tree-spined stickleback the constraint of locus specificity can be neglected, because the different MHC loci do not

show locus specific patterns due to concerted evolution [62, 63]. Previous studies have used different approaches to study the MHC class IIB genes of the stickleback and developed a number of different primer pairs [11, 15, 65], however none of the primer pairs alone amplifies all present sequence variants. Therefore we strove to develop a primer pair that amplifies all exon 2 alleles simultaneously and, together with the higher resolution of RSCA, would provide a fast and reliable genotyping technique for future research on MHC in sticklebacks.

Our criteria for the primer pair used for RSCA genotyping were, first, that they align in a conserved region to avoid primer bias of the detected allele pool, second, that they comprise as much as possible of the highly variable exon2 and third, that both primers align within the exon2 to avoid any intron length variation of the amplicon, which would inhibit hybridisation with the reference.

Due to the high sequence variation in exon 2 of the MHC class II loci [61, 62, 66], the first criteria produces a certain challenge to the primer design. However, using recently published new sequence information [15, 62, 63] and the stickleback genome (http://mar2008.archive.ensembl.org/Gasterosteus_aculeatus), we could develop a new primer pair for the Three-spined stickleback's MHC IIB. The new forward primer: GAIEx2startF (5'-GTC TTT AAC TCC ACG GAG CTG AAG G -3') is optimised for the three-spined stickleback and aligns in a fully conserved region in the beginning of exon 2 (Fig 1). The new reverse primer GAIExon2R_RSCA (5'-ACT CAC CGG ACT TAG TCA G -3') spans the exon 2 – intron 2 boundary and lies in a completely conserved region as well (Fig 1). The partial connection to the exon 2 still avoids any length polymorphisms, which occur frequently in the rest of the intron 2 and would inhibit the hybridisation process necessary for RSCA typing. Although the new reverse primer spans the exon-intron boundary, it can nevertheless be used for expression studies, because it aligns with more than two thirds in the exon 2 and the first base pairs of intron 2 and exon 3 are highly similar, leaving only 2 fully conserved mismatches at the 5'-end in exon 3. In combination with the new reverse primer this combination produces a fragment of 247 base pairs (203 bp without primers) and spans 88 % of the entire exon2. These primers amplify all currently known MHC IIB loci. For simplicity reasons we refer to different sequence variants as alleles, although they may stem from different loci.

- **Selection of reference alleles for RSCA typing**

Selection criteria for the fluorescent labelled references (FLRs) were: Firstly, they should represent sequence variants that do not occur in the screened populations. Secondly, they should not be too genetically distant to assure reliable hybridisation. And thirdly, to increase resolution, the individual reference sequences should be as dissimilar from each other as possible. Due to the trans-species polymorphism of the MHC [67] it is even possible to use reference sequences from closely related species [33], but this increases the risk that certain alleles do not hybridize, which again increases with the complexity of the template (number of

alleles) because of competition between alleles during the hybridisation reaction. In our case we tested nine sequence variants of three-spined sticklebacks from a West Canadian population, which had been cloned previously (TBH Reusch & T Reimchen, unpublished data) and due to long divergence time [68] are unlikely to carry any Northern European alleles. Due to the existence of a comprehensive sequence database of more than 120 sequence variants that had been collected over the years [15, 62, 63](TBH Reusch, KM Wegner, C Eizaguirre & TL Lenz, unpublished data) we were able to compare the genetic distance of the Canadian alleles with the average of our local populations and could choose candidates that differed from each other more, but from the local alleles on average less than the population average. Additionally we estimated the distribution of the genetic distance between each reference and all the known alleles from our local populations to choose the flattest and broadest distribution (see Fig 2 for examples). A flat distribution promises the highest resolution of alleles, because genetic distance and molecule mobility in the gel matrix are highly correlated due to the three dimensional structure of the heteroduplex that increases in complexity with increasing mismatches between the reference and the target strand (data not shown). A flat distribution of the genetic distances between FLR and the allele pool therefore leads to a broader distribution of allele peaks and avoids overlap between them.

- **General RSCA protocol**

- o Production of fluorescent labelled reference strands

The templates for the FLRs were plasmids containing a single sequence variant that was obtained through cloning three-spined sticklebacks from a Canadian population (TB Reusch & T Reimchen, unpublished data). The plasmids were diluted 1:1000 and amplified with the same primer pair as the fragment that was to be typed, except that the forward primer was fam-labelled. The PCR mix for a 50 µl reaction contained 5 µl diluted plasmid, 1x GeneAmp PCR Buffer II (Applied Biosystems), 5 mM MgCl₂, 50 µM of each dNTP, 0.5 µM of each primer and 2.5 units of AmpliTaq Gold (Applied Biosystems). The following PCR program was used: 95°C for 10 minutes to activate the hot-start polymerase, 33 cycles of 94°C for 30 seconds, 58°C for 30 seconds and 72°C for 60 seconds with a final extension step of 72°C for 5 minutes. We ran our programs on the thermal cyclers PC-200 (Bio-Rad, Munich, Germany) or LabCycler (SensoQuest, Göttingen, Germany). The PCR products were directly purified with the NucleoSpin Extract II Kit (Macherey Nagel, Düren, Germany) to eliminate the labeled primer and eluted in 100 µl HPLC grade water (room temperature, Mallinckrodt Baker, USA). The purified FLRs were kept for days in the fridge or weeks at -20°C.

- Amplification and hybridization

Target of the genotyping was the exon 2 of the MHC class II loci amplified from genomic DNA of individual three-spined sticklebacks. Genomic DNA can for instance be extracted from the tip of a dorsal spine, a part of the tail fin or from fresh or ethanol preserved muscle tissue. Here we used preserved muscle tissue and the DNeasy Blood and Tissue kit (Qiagen, Hilden, Germany). For amplification of the exon 2 we used the newly developed primer pair GAIEx2startF and GAIExon2R_RSCA (247 bp product). The number of PCR cycles should be as low as possible to avoid formation of artefacts [20, 69]. A reaction of 25 µl contained app. 90 ng of DNA, 1x GeneAmp PCR Buffer II, 5 mM MgCl₂, 50 µM of each dNTP, 0.5 µM of each primer and 1 unit of AmpliTaq Gold polymerase. The following PCR program was used: 95°C for 10 minutes to activate the hot-start polymerase, 27 cycles of 94°C for 30 seconds, 58°C for 30 seconds and 72°C for 60 seconds with a final extension step of 72°C for 5 minutes. A reconditioning PCR step against artefact formation could be omitted here, because heteroduplexes after the last PCR cycle, the source for such artefacts, are eliminated with the following hybridisation reaction of target and FLR [20]. We ran our programs on the thermal cyclers PC-200 (Bio-Rad) or LabCycler (SensoQuest).

Subsequently the appropriate amount of PCR product is mixed with the respective FLR. This has to be adjusted for each FLR, because the hybridisation efficiency is altered by the average genetic distance between target alleles and FLR as well as GC content of the sequences. The ratio depends also on the concentration of the PCR product and the FLR. In the end, a good ratio should give equal heights of homo- and heteroduplexes. In our case we used 6 µl PCR product for two of the FLRs and 8 µl for the third in combination with 1 µl of the respective FLR. The hybridisation starts with a denaturation at 95°C for 10 minutes, then the heteroduplex formation is facilitated by a slow cooling ramp of 2°/sec to 55°C, which is subsequently held for 20 minutes. A final cooling step of 4°C for 15 minutes assures stabilisation of the heteroduplexes. This hybridisation product is stable for several hours at 4°C and for several days at -20°C.

- Capillary electrophoresis

The separation of heteroduplexes was performed on an ABI PRISM 3130xl Genetic Analyzer (Applied Biosystems) with a 36 cm capillary. A 5% Conformation Analysis Polymer (CAP, Applied Biosystems) provided the non-denaturing matrix in which the heteroduplexes migrate according to their tertiary structure. We used a 5% polymer: 5 g 9% CAP (prewarmed to room temperature), 2.16 g Urea (Sigma-Aldrich, Steinheim, Germany), 0.95 g HPLC grade water and 0.9 g 10x Running Buffer (Applied Biosystems). For each sample 1.5 µl of the hybridisation product was mixed with 0.3 µl GS1000 Rox size standard (Applied Biosystems) and 9.7 µl HPLC grade water. The internal size standard ensures proper alignment of

heteroduplex peaks and minimizes between-run variation. The running conditions were: 18°C run temperature, 15 kV injection voltage, 15 sec injection time, 10 kV run voltage. The hybridisation products of each FLR had to be run separately, otherwise the antisense strand of one FLR would hybridise with the labelled strand of another FLR and produce a heteroduplex peak that could not be differentiated from real alleles.

- Plasmid library for sequence specific genotyping

A plasmid library of MHC class II exon 2 sequence variants has been collected over the years from cloned three-spined sticklebacks (TBH Reusch, KM Wegner, C Eizaguirre & TL Lenz, unpublished data). It comprises currently 83 distinct variants that differ to varying extents (1 to 47 of 203 bp). Of the 3403 potentially possible allele combinations in a pairwise comparison, only 0.7% differ by less than 3 bp (17 by 1 bp and 8 by 2 bp). We recorded mobility values for each sequence variant with each of the three FLRs. This was done four times independently using different polymer lots to determine the run-to-run variability of the mobility values. To estimate the resolution and specificity of the new typing method, we compared the difference in mobility values of alleles pair-wise in all 3403 possible combination. Those allele pairs that differed for all three FLRs in their mean by less than their combined standard deviation were assigned to be undistinguishable.

- RSCA typing procedure

MHC II exons 2 of individual three-spined sticklebacks were amplified with the newly developed primers and processed according to the protocol outlined above. Mobility values for each heteroduplex peak were recorded after alignment of the internal size standard. Using the mobility values from the three FLRs and the library with mobility values of the collected alleles, we could assign allele identity to the heteroduplexes (see Fig 5 for an example). However, identity of an allele was only called if all three corresponding mobility values (± 1 SD) from the library were found in the individual. In cases where not all three values from the allele library could be found in an individual, we marked the heteroduplex peak as new allele. Individuals with such unknown heteroduplexes were subsequently cloned to identify the sequence of the new allele.

- Cloning of selected fish to verify RSCA typing

To verify our RSCA genotyping procedure, we compared it with the result of 23 thoroughly cloned and sequenced sticklebacks from different locations and time points. Ten of these fish had been genotyped previously to establish a reliable amplification and cloning protocol for multi-locus templates [20]. This involved several steps to avoid artefact formation during PCR and cloning and the sequencing of on average 89 clones per individual. This study used two

established primers, which amplify a shorter fragment of the exon 2 than the newly developed primer pair.

We then cloned an additional set of 13 three-spined sticklebacks, some of which, according to RSCA typing, possessed so far unknown alleles. For the amplification and cloning of these fish, we followed the protocol in Lenz & Becker [20], with the exceptions that we used the newly developed primer pair, only two independent amplification reactions and picked 48 clones per fish for sequencing. 48 clones is a threshold that we determined using accumulation curves on data of the first cloning set and which gives 99.99% confidence to have typed all present sequence variants (Fig 3).

- **Applied software**

For alignment and estimation of genetic distance of sequences, we used BioEdit 7 [70]. Chromatograms were aligned according to the internal size standard and analysed with GeneMarker 1.6 (SoftGenetics, PA State College, USA). Due to the non-denaturing property of the polymer, the supplier-provided values of the size standard did not match the peak pattern and therefore the values for the size standard peaks were assigned artificially starting with 1000 for the longest fragment.

The software Primer 6 [71] was used to calculate accumulation curves based on clone data from the previous cloning [20]. To get a bootstrap estimate of confidence, we resampled 999 times the clones for an individual with five alleles.

We used the ScoreCons online server [72] to determine variation for amino acid residues of the exon 2. The software MultiLocus 1.22 [73] was used to estimate linkage disequilibrium between detected alleles and 1000 randomizations were run.

Results

- **Plasmid library**

The three mean mobility values for each of the 83 allele variants averaged over at least four independent runs are shown in Fig 4. To estimate the resolution of the RSCA typing method, we compared all 83 alleles pair-wise. The mean mobility difference between alleles was 46.5, 42.3 and 27.3 units respectively for the three FLRs. In comparison, the average standard deviation of the independently obtained mobility values of an allele was 1.2, 1.0 and 1.4 units respectively. In seven (0.2%) of 3403 possible allele pairs, the two alleles were not distinguishable according to our definition outlined above. Six of them differed by 1 bp and one by 2 bp. The other 11 pairs with 1 bp and seven with 2 bp difference could be distinguished by using the three different FLRs (Fig 4).

- **RSCA typing of selected fish**

The three mean mobility values for each of the 83 allele variants averaged over at least four independent runs are shown in Fig 4. To estimate the resolution of the RSCA typing method, we compared all 83 alleles pair-wise. The mean mobility difference between alleles was 46.5, 42.3 and 27.3 units respectively for the three FLRs. In comparison, the average standard deviation of the independently obtained mobility values of an allele was 1.2, 1.0 and 1.4 units respectively. In seven (0.2%) of 3403 possible allele pairs, the two alleles were not distinguishable according to our definition outlined above. Six of them differed by 1 bp and one by 2 bp. The other 11 pairs with 1 bp and seven with 2 bp difference could be distinguished by using the three different FLRs (Fig 4).

- **Cloning and comparison to RSCA**

Cloning and sequencing of 23 individuals revealed 86 alleles, 27 of them being distinct. All sequences produced strongest NCBI-BLAST hits with stickleback MHC IIB exon 2 variants. 16 of the 27 alleles were already known in GenBank (Suppl. Tab 1) and the remaining ones were also submitted (GenBank accession numbers: FJ360531 - FJ360541). An additional very divergent sequence variant (GenBank: AF395709), which has been described before [62], was detected by cloning as well as RSCA typing to be present in every individual and due to sequence conservation was not further addressed in our comparison. This sequence, which can also be found in the genome sequence of the Alaskan stickleback, potentially stems from an invariant MHC locus, which could for instance have an antigen processing function, just as the invariant H2-M locus in mice [74].

The remaining alleles differed in 1 to 46 nucleotides with a mean of 26. The results of the cloning confirmed the previous RSCA typing completely in 22 out of 23 individuals for allele number and allele identity, i.e. known alleles were recognised and unknown alleles were called as new and differentiated from each other. In one individual a known allele (Neu51) was not recognised and called as new, although it was present in the allele library and had even been recognised in another fish of the same set (Suppl. Tab 1). This is however a conservative ambiguity that would have been resolved by following the proposed RSCA typing protocol, i.e. cloning the respective individual to resolve the supposedly new sequence variant. In this set of 23 individuals therefore 16240 bases were typed correctly and another 1218 bases (5 different alleles in 4 individuals) were scheduled for cloning by the proposed protocol.

The detected alleles showed strong linkage disequilibrium ($I_A = 0.38$, $p < 0.001$). All alleles that occurred in more than one individual showed obvious haplotypic patterns with one to three variants per linkage group (Suppl. Tab 1). In total, 15 distinct haplotypes could be differentiated, two of which shared one allele.

We also RSCA-typed the first ten fish, which had been cloned with a different primer pair in the earlier project, with this same primer pair to enable direct comparison between methods. The same number of alleles per individual could be observed, which shows that the RSCA typing protocol is independent of the used primer pair.

Discussion

In this study we developed a protocol for reliable genotyping of the highly polymorphic MHC class IIB genes of the Three-spined stickleback using Reference Strand-mediated Conformation Analysis. To verify the results obtained from RSCA typing, we cloned and sequenced a total of 23 individuals to saturation. The congruence between RSCA and cloning in number as well as identity of detected alleles shows the reliability of the new typing protocol. While the process of amplification, cloning, plasmid extraction, sequencing and alignment can take several weeks and is relatively laborious, our RSCA protocol can handle hundreds of fish in a number of days with not much more than the cost for PCR and some sequencer runs. Incorporating a library of collected sequence variants, we were able to unambiguously identify the sequence information underlying each peak or assign new alleles if applicable. Only in one case out of 86 alleles was an allele assigned as new, although its sequence was already known. This is due to the adopted stringent typing procedure and a conservative error, which was resolved by following the typing protocol, i.e. cloning and sequencing this individual.

The identification of each allele to the sequence level is a clear advantage of the new RSCA protocol over the previously used CE-SSCP typing. This sensitivity will enable future comparisons of allele pools from different populations and over different time points as well as analysis of evolutionary important parameters such as residue specific patterns of adaptation or the effect of genetic distance between alleles and individuals. Recent studies have revealed intriguing insights into the functional aspects of sequence and even residue specific differences for mate choice [26, 28, 29], parasite resistance [75] and adaptive divergence of species [27].

The relatively high number of unknown alleles in the 23 analysed individuals is due to the fact that some of these individuals were chosen for cloning, because they contained new alleles. From our experience so far, the average fraction of individuals with new alleles lies in the range of 5-7%, i.e. only this fraction of a given sample set has to be cloned to get the complete sequence information for all individuals. However, these numbers are highly linked to the completeness of the allele library that is used for the proposed RSCA genotyping protocol. As soon as new populations with divergent allele pools are sampled, the fraction of individuals with new alleles will increase again.

In addition to the RSCA protocol, we also developed a new primer combination, which is more universal than the ones previously used, because it amplifies all present alleles in a single reaction. These primers amplified reliably in the local populations as seen in the here presented data and followed haplotypes over two generations in a large breeding project of several families from two different populations (data not shown). It remains to be tested whether they work satisfyingly across all stickleback populations from the Northern hemisphere, considering the divergence between European populations [68] and even more between the Atlantic and the Pacific clade [76]. The currently available MHC sequence information from the genome of an Alaskan individual and sequences of some individuals from British Columbia / Canada (TBH Reusch & T Reimchen, unpublished data), however, support the universality of our new primers. Using these primers in the current study, we found a median of 4 alleles over 23 individuals, which is slightly lower than the previously reported 5.8 alleles per individual detected by CE-SSCP [77]. This might be due to the limited number of individuals in our study, but it might also indicate that the previously used combination of two reverse primers [15] slightly overestimated the total MHC diversity. This would fit to a recent finding, which estimates the number of MHC class IIB loci in the Three-spined stickleback to only 2-4 [62].

We did not test, whether all the detected alleles are expressed, however, a previous screen of several families revealed that over 90 % of the alleles detected by CE-SSCP are expressed [48]. In combination with the new typing technique, it remains to be tested whether all alleles are expressed in all organs, or whether there is locus specific transcription regulation. This becomes even more interesting in the light of previous results, which show substantial variation in overall MHC expression between families, organs and even haplotypes [78]. Such transcriptional variation receives increasing attention by evolutionary biologists, as it provides a potentially underestimated source for adaptability in natural populations [79-83].

The first of the two only studies employing RSCA in non-mammalian species so far directly compared RSCA with SSCP typing, when genotyping the MHC class IIB locus in Lake trout [*Salvelinus namaycush*, 42]. In the end the authors favoured SSCP over RSCA, because they found a slightly higher overall number of alleles with SSCP. However the existence of these additional alleles was not verified via sequencing. Moreover, the Lake trout carries only one MHC IIB locus, which limits the number of alleles to be detected to two per individual. The authors of the second study developed an RSCA typing protocol for both MHC class I and II in the Red jungle fowl and addressed two loci at a time respectively [43]. The authors show congruency with cloning in eight individuals and conclude that RSCA is a reliable technique for MHC typing in the Jungle fowl.

In many species, the MHC consists of several loci in both classes [84], which makes the allele pattern more complicated and increases the chance of overlapping signals using SSCP, a

problem that occurred already with the single locus in the Lake trout [42]. Here, RSCA has a substantial advantage over other indirect typing techniques, because it provides several mobility values per allele, reducing the chance of two overlapping allele values and increasing resolution.

Reproducibility is also a major concern for indirect genotyping techniques and can be confounded by polymer lot variation, temperature fluctuation and other factors [85]. Therefore we measured the variation of mobility values for each allele across independent runs over several months. To test realistic laboratory conditions, we used different polymer lots, the most likely source of between run variation, and a capillary that is in turns used also for fragment analysis with POP-4™ polymer (Applied Biosystems). By this we could show that between-run variation with RSCA is limited and can be overcome by the advantage of getting several mobility values from the different labelled references. We conclude that three different well-chosen reference strands are enough to differentiate 99.8% of the 3403 potentially possible allele pairs and still 72% (18/25) of those allele pairs that differ only by one or two nucleotides (see Fig 4). Employing more reference alleles would probably also differentiate the last allele pairs, but we consider a resolution of 99.8% as high enough. Nevertheless, regular re-runs of known alleles / allele libraries are advisable to keep the mobility values “up to date” and counteract unreported chemistry changes by suppliers.

An interesting advantage of indirect genotyping techniques compared to cloning and sequencing is their quantitative aspect. The clone picking, especially with low numbers of clones, produces an arbitrary snap shot of the real molecule picture, whereas in SSCP, DGGE or RSCA the relative quantity of sequence variants is reflected directly by peak height or band intensity. Therefore these indirect techniques are less affected by artefact formation than cloning, where artificial sequences can lead to alteration of analysis [20]. This is especially true when picking only a small number of clones, because then each randomly picked clone represents hundreds of thousands of molecules and initially low represented artefacts or contamination might be overestimated.

A new observation due to the high resolution in allele detection by RSCA is the fact that alleles seem to occur in linked haplotypes, differ in the number of sequence variants per haplotype and in one case share an allele between haplotypes. This provides a hint for strong linkage disequilibrium between loci and low but yet occurring recombination in nature, an observation that again fits to previous insights into the organisation of the MHC region in the stickleback [63]. More importantly however, it indicates variation in the number of loci between haplotypes and reveals a phenomenon that has already been described for other species [86-90]. This finding would explain the earlier reported large variance in allele numbers among individuals [77] and reveals a potential mechanism of adaptation to changing pathogenic environments, which has first been termed by Klein *et al.* [91] as 'The Accordion Model of MHC Evolution' and

was later elaborated by Nei *et al.* [9] to the Birth-and-Death-Model of Evolution. Nevertheless these findings need further research on the genomic level, including more thorough analysis of the haplotype specific chromosomal organisation.

Conclusion

Here we present a new RSCA genotyping protocol for the highly polymorphic MHC genes of the Three-spined stickleback, which in combination with a comprehensive allele library provides sensitive and reliable allele data to the sequence level. Verification of the RSCA typing by cloning and sequencing shows high congruency between both techniques. Together with new insights into the polymorphic MHC of the Three-spined stickleback, an emerging model system, this offers a resource for researchers to address questions of host-parasite co-evolution, local adaptation and ecological speciation.

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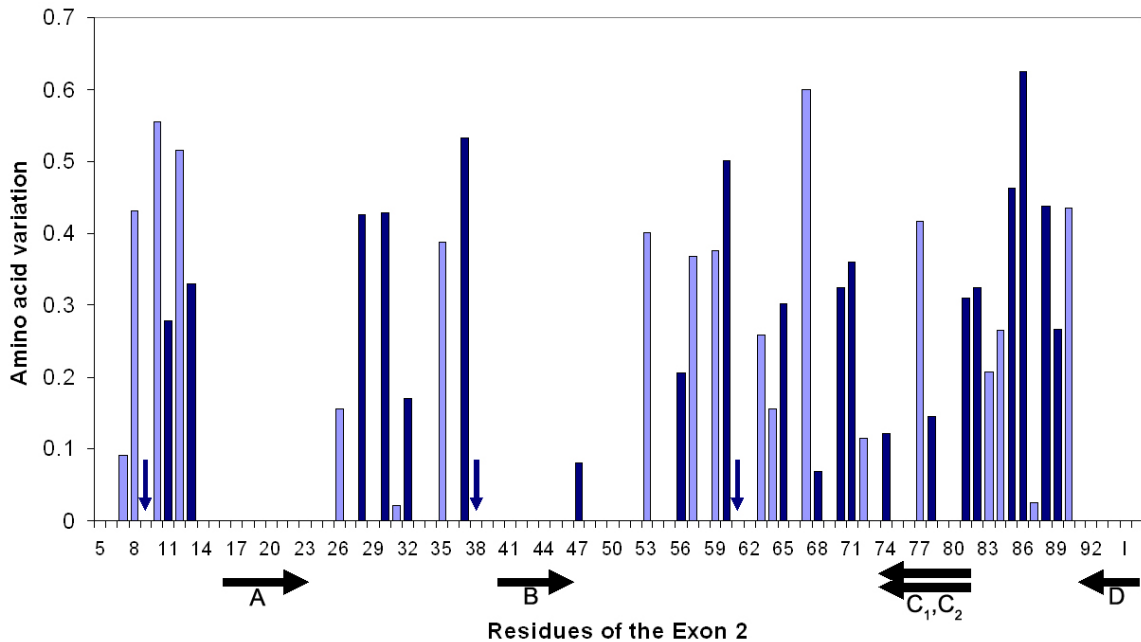


Fig 1: Variation score for amino acid residues of the exon 2 based on 31 local sequence variants of the full exon 2 and adjacent intron 2. The variation for each residue is based on the number and frequency of substitutions and is calculated as $y = 1 - \text{Valdar01 score}$, as determined with the Scorecons server by Valdar (2002). Dark blue bars and arrows indicate residues involved in antigen binding according to Brown *et al.* (1993). Black arrows on the bottom show positions of the available primers for the Exon 2: A (GAIEx2startF) and D (GAIExon2R_RSCA) are the primers developed for this study; B and C₁,C₂ are the primers used in previous CE-SSCP typings of the stickleback MHC (Binz *et al.* 2001b; Reusch *et al.* 2001a). For visual reasons, the variation plot was extended into the adjacent intron 2 for 6 bases, although an amino acid variation score is not applicable on intronic sequence. Nevertheless these 6 base pairs are fully conserved in all known sequences so far.

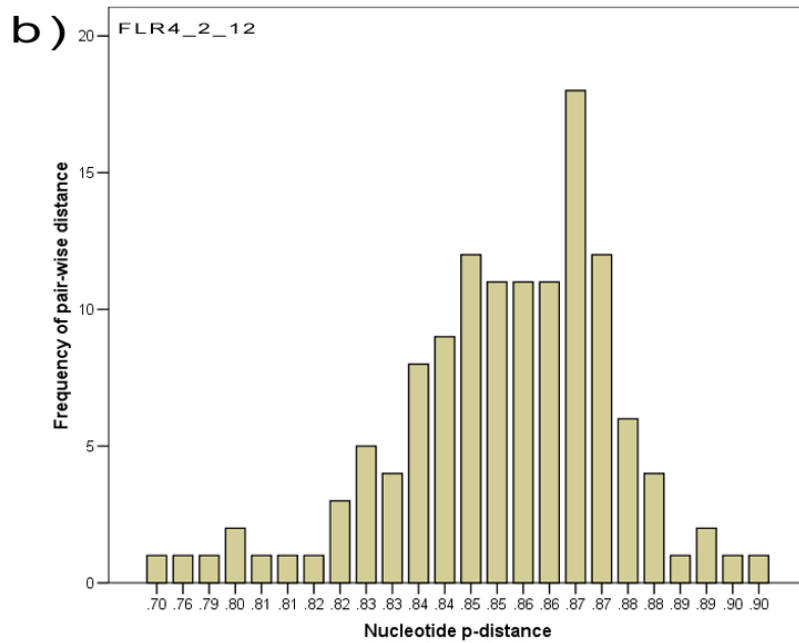
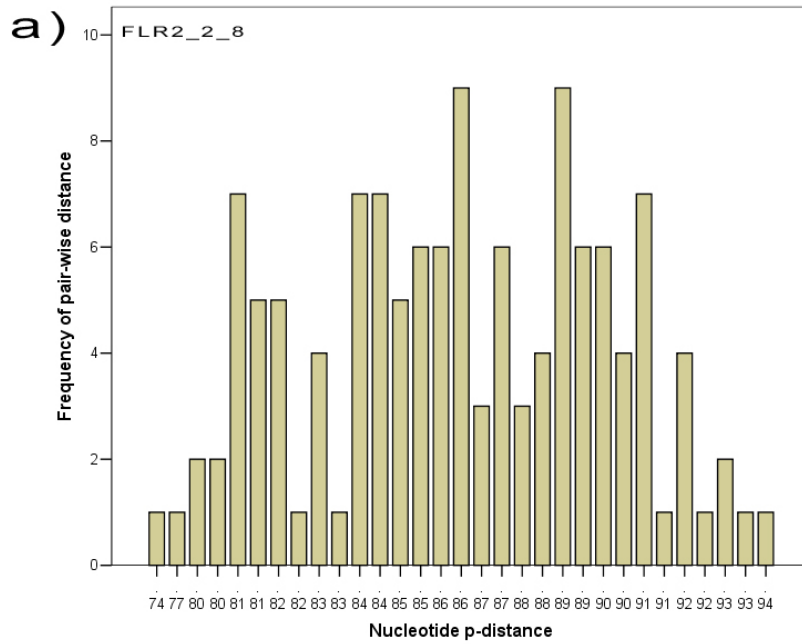


Fig 2: Distribution of genetic distances between alleles in the collected library and a) a suitable reference allele (FLR) with a broad distribution of genetic distances, and b) an unsuitable FLR with a narrow distribution.

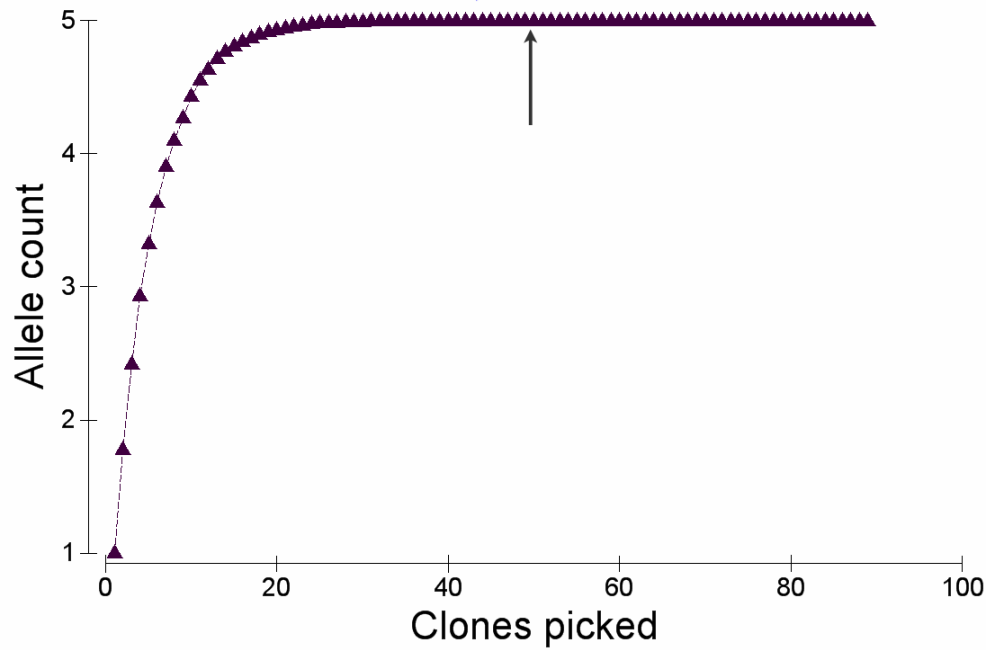


Fig 3: Accumulation curve for the number of clones sequenced to surely detect all alleles of an individual. This curve was bootstrapped 999 times on the clone data of an individual with five alleles. The arrow shows the threshold for 99.99 % detection of all present sequence variants (calculated for an individual with 5 alleles).

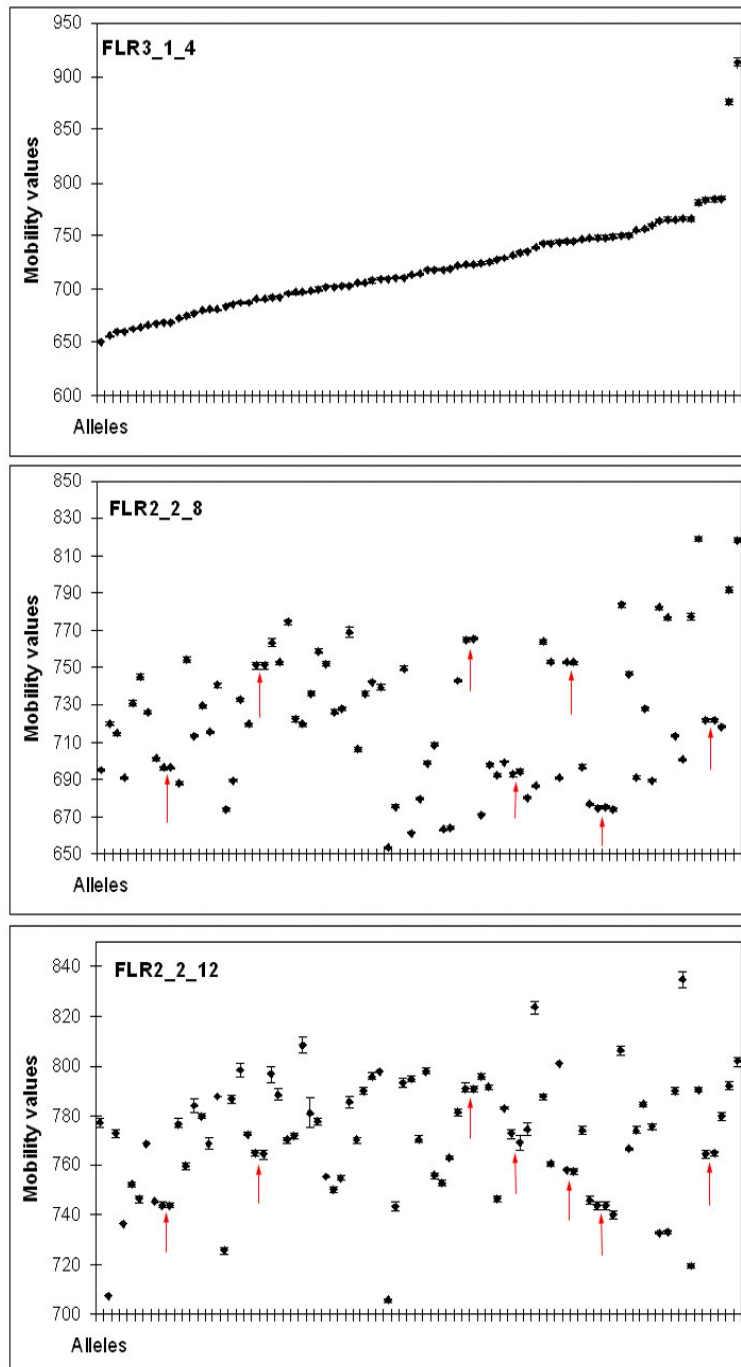


Fig 4: Mobility values of 83 alleles with the selected three FLRs. Mean and SD of each allele from at least four independent runs are shown. Red arrows indicate the allele pairs that can not be differentiated with the three FLRs. Alleles are presented in the order of mobility with the reference FLR3_1_4.

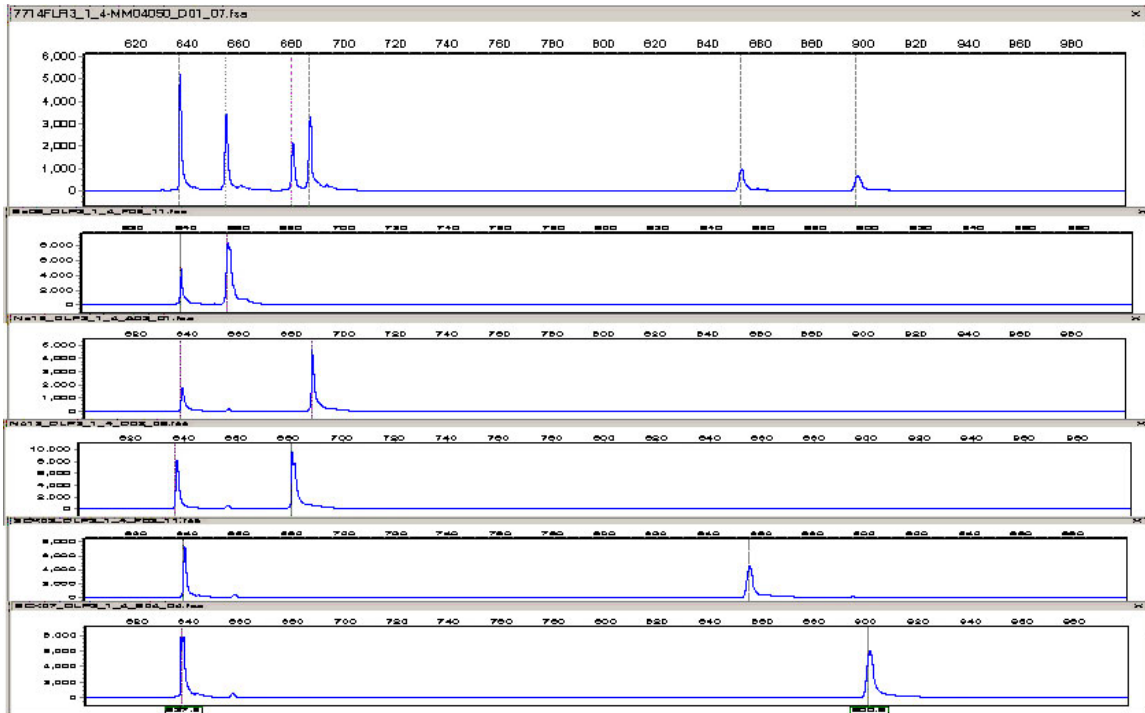


Fig 5: RSCA chromatogram of an individual with 5 alleles hybridised to one of the three FLRs. Below are shown the chromatograms of the corresponding five plasmids from the allele library hybridised to the same FLR.

Appendix II

This part contains supplementary information of Chapter II.

Supplementary Table 1: Number of reproducing sticklebacks recaptured from each enclosure at the end of the experiment (from eight fish per sex and enclosure originally introduced) and mean number of analysed eggs assigned to them.

	N _♂ SURVIVED AND REPRODUCED	N _♀ SURVIVED AND REPRODUCED	MEAN N _♂ EGGS +/- ST DEV	MEAN N _♀ EGGS +/- ST DEV
Enclosure 1	7	5	38.4 +/- 26.2	49.6 +/- 16.4
Enclosure 2	5	5	69.8 +/- 47.6	66.8 +/- 24.8
Enclosure 3	6	4	47.6 +/- 44.6	45.0 +/- 11.9
Enclosure 4	2	3	52.5 +/- 19.1	44.3 +/- 12.5
Enclosure 5	2	6	25.0 +/- 9.9	60.0 +/- 17.0
Enclosure 6	3	5	81.3 +/- 44.6	44.8 +/- 34.5

Mean N_♂ eggs +/- St dev: Mean number of egg fathered per male +/- standard deviation.

Mean N_♀ eggs +/- St dev: Mean number of egg mothered per female +/- standard deviation.

Supplementary Table 2: This table describe the PCR protocols for the microsatellites multiplex PCRs used to identified the fish and for parenthodd analysis,

Table 1: Multiple primer PCR protocols used for microsatellite typing.

PCR 1	PCR2	PCR3	PCR4	PCR5
10 x buffer	10 x buffer	10 x buffer	10 x buffer	10 x buffer
1.00	1.00	1.00	1.00	1.00
dNTP's	dNTP's	dNTP's	dNTP's	dNTP's
1.00	1.00	1.00	1.00	1.00
% BSA	1 % BSA	MgCl2	MgCl2	MgCl2
1.00	1.00	0.30	0.30	0.30
MgCl2	MgCl2	STN 2 f	STN 36 f	STN 24 f
0.30	0.30	0.30	0.30	0.30
Stich 5196 f	Stich STN18 f	STN 2 r	STN 36 r	STN 24 r
0.10	0.12	0.30	0.30	0.30
Stich 5196 r	Stich STN18 r	STN 170 f	STN 114 f	STN 67 f
0.10	0.12	0.30	0.30	0.25
Stich 4170 f	Stich STN32 f	STN 170 r	STN 114 r	STN 67 r
0.08	0.08	0.30	0.30	0.25
Stich 4170 r	Stich STN32 r	STN 174 f	STN 167 f	STN 185 f
0.08	0.08	0.30	0.45	0.30
Stich 1125 f	Stich STN75 f	STN 174 r	STN 167 r	STN 185 r
0.10	0.17	0.30	0.45	0.30
Stich 1125 r	Stich STN75 r	Taq (Invitex)	Taq (Invitex)	Taq (Invitex)
0.10	0.17	0.05	0.05	0.05
Stich 1097 f	Stich STN 84 f	Water	Water	Water
0.40	0.08	4.85	4.55	4.95
Stich 1097 r	Stich STN 84 r			
0.40	0.08			
Stich 7033 f	Taq (Invitex)			
0.35	0.05			
Stich 7033 r	Water			
0.35	4.75			
Taq (Invitex)				
0.05				
Water				
3.59				

All quantities are in µl. The colors represent the label used. Working concentration of the primers: 5 pmol.

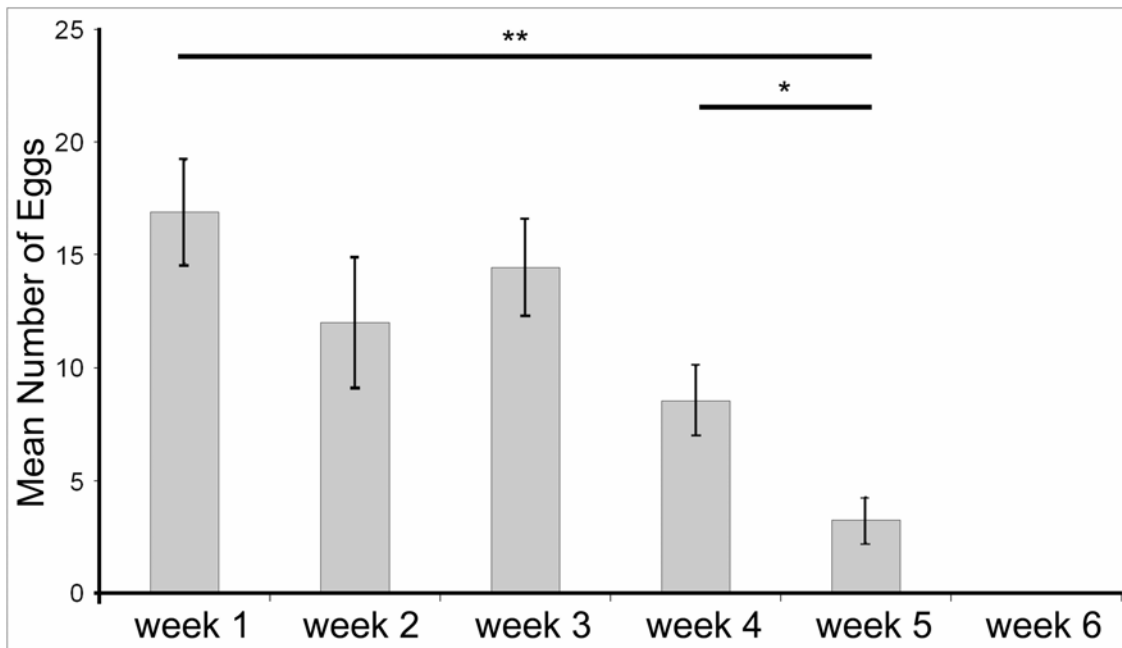
Supplementary Table 3: This table describes the PCR conditions and cycles for each multiplex PCR performed.

	PCR 1	PCR2	PCR3	PCR4	PCR5
Original Denaturation	3 min at 94°C	3 min at 94°C	2 min at 94°C	2 min at 94°C	2 min at 94°C
Denaturation	1 min at 94°C	0.45 min at 94°C	0.45 min at 94°C	0.45 min at 94°C	0.45 min at 94°C
Annealing	1 min at 58°C	0.45 min at 56°C	0.45 min at 56°C	0.45 min at 56°C	0.45 min at 56°C
Extension	1 min at 72°C	1 min at 72°C	0.45 min at 72°C	0.45 min at 72°C	0.45 min at 72°C
Final extension	None	none	20 min at 72°C	20 min at 72°C	20 min at 72°C
Number of cycle	27	26	27	27	27

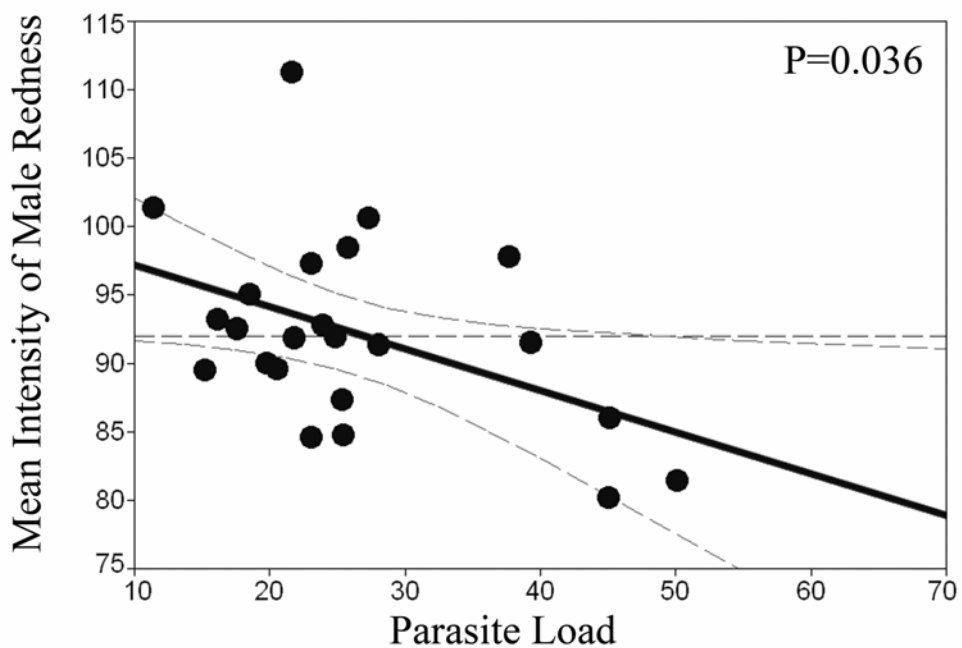
Supplementary Table 4: Table summarizing the prevalence (percentage of infected hosts) and the mean intensity (number of parasite individuals of a given species per infected host) of all recorded parasites in the 53 dissected fish.

	Prevalence (%)	Mean Intensity
<u>Protozoa:</u>		
<i>Trichodina sp.</i>	88.46	18.62
<i>Apiosoma sp.</i>	9.62	5.50
<i>Ichthyophthirius multifiliis</i>	11.54	2.33
<u>Monogenea:</u>		
<i>Gyrodactylus sp.</i>	28.85	17.80
<u>Digenea:</u>		
<i>Diplostomum sp. (total)</i>	100.00	15.98
<i>D. pseudospathaceum (lab infection)</i>	100.00	5.06
<i>Cyathocotyle prussica</i>	98.08	4.35
<i>Echinochasmus sp.</i>	98.08	4.83
<i>Apatemon cobitidis</i>	71.15	4.70
<i>Tylodelphis clavata</i>	98.08	6.93
<i>Phyllodistomum folium</i>	25.00	3.75
<u>Cestoda:</u>		
<i>Valipora campylancristota</i>	84.62	11.93
<i>Proteocephalus filicolis</i>	86.54	6.87
<u>Nematoda:</u>		
<i>Anguillicola crassus</i>	69.23	1.64
<i>Contraecum sp.</i>	13.46	1.00
<i>Camallanus lacustris</i>	86.54	4.33
<i>Raphidascaris acus</i>	71.15	2.97
<u>Acanthocephala:</u>		
<i>Acanthocephalus clavula</i>	34.62	2.00
<i>Acanthocephalus lucii</i>	78.85	1.00
<u>Crustacea:</u>		
<i>Argulus foliaceus</i>	15.38	1.91
<i>Ergasilus sp.</i>	9.62	1.00
<u>Mollusca:</u>		
Glochidia	23.08	1.00

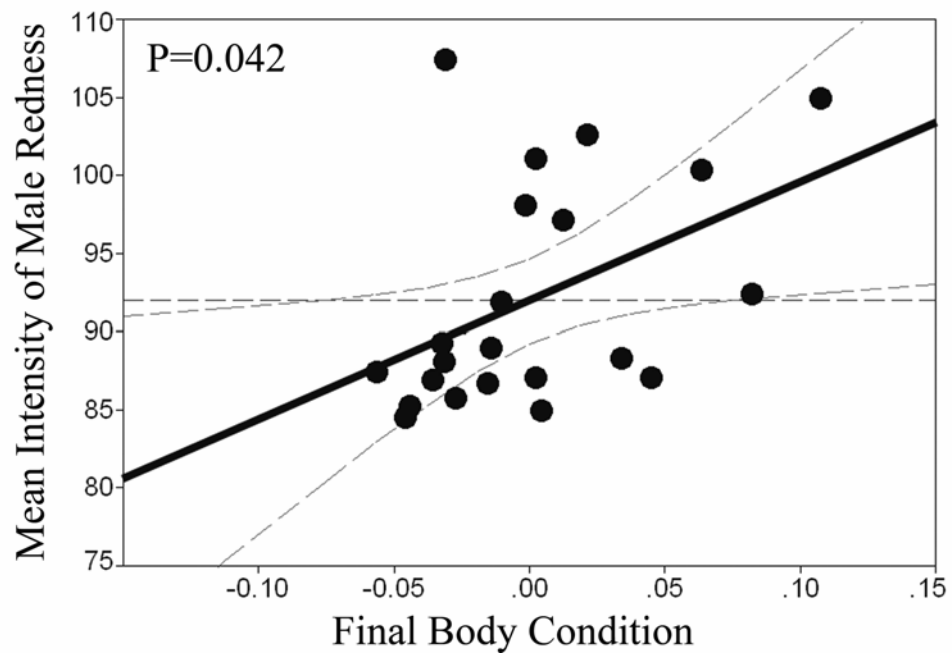
Supplementary Figure. 1: Mean (+/- S.E.) representative number of eggs collected weekly during the experiment. The mean decreased significantly until the last collecting day, indicating that the reproductive period has finished until week 6. Significant differences between the first and last week, and between the last two are depicted by solid horizontal lines.



Supplementary Fig. 2: Graph representing the mean intensity of the red coloration of males' throat in relation with individual parasite load. P value of the correlation is indicated on the graph.



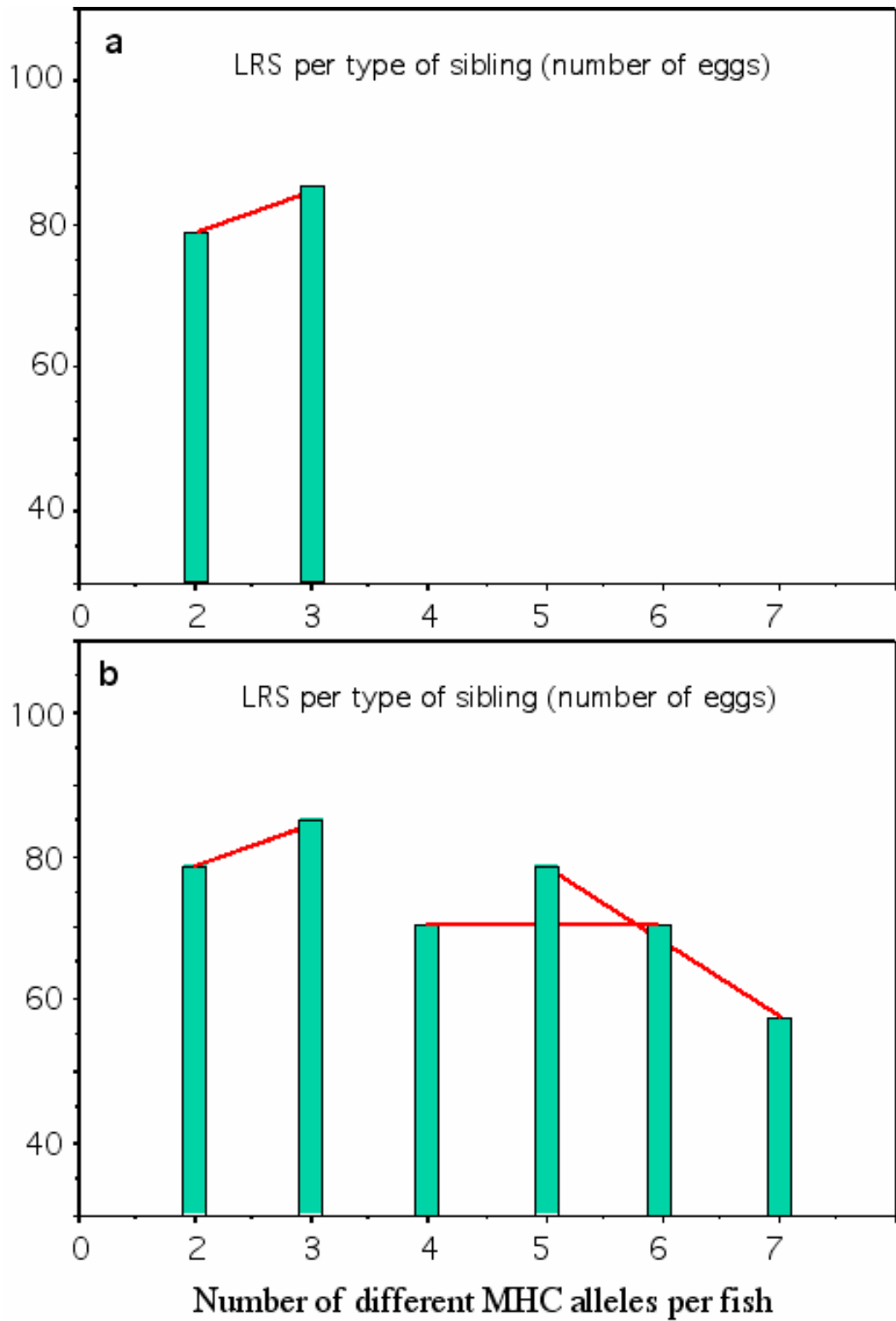
Supplementary Fig. 3: Representation of the correlation between male breeding coloration and standardized final body condition. P value of the correlation is indicated on the graph.



Supplementary document 4: Testing for immunogenetic optimality by combining several sibships

This is a fictitious example to show how we can test for an optimal number of different MHC class IIB alleles that maximises lifetime reproductive success. The logic of this procedure is the same as in similar analyses for immunogenetic optimality (Wegner et al. 2003a) or optimising MHC heterozygosity for offspring through mate choice (Aeschlimann et al. 2003; Milinski 2003). For the present example we combine the data from several sibships each confined in one separate enclosure. Let us assume for simplicity that we find two different genotypes with respect to number of different MHC class IIB alleles in each sibship. Of course more than two genotypes may exist, but the analysis would be similar. Let us assume the two genotypes of sibship one have 2 and 3 different MHC alleles, respectively. Now we determine the mean LRS for each of the two genotypes (supplementary Fig. 4a). If we connect the two values for LRS we find a line with positive slope. So the potential optimum must be higher than 2.5 alleles, which is the average of these two options. In another sibship we find 2 genotypes with 5 and 7 alleles, respectively. Now the slope of the line connecting these two LRS-values is negative (supplementary Fig. 4b, right). This suggests that the potential optimum is lower than 6 alleles, which is the average of these two values. If the average of two options is the optimum number of alleles, the slope of the connecting line should be zero, as in another sibship (supplementary Fig. 4b, middle). We can do this analysis with 6 fictitious sibships. Each sibship gives us a pair of data, namely the average combined allele number and the slope of the line connecting the respective two LRS-values. We can now take each data pair per sibship to a new figure, i.e. the average allele number as x-value and the respective slope as y-value. Now we calculate the linear regression through these 6 slope values (as in Fig. 2b in the main text). The optimum exists if the new regression line has slope, which is significantly different from zero. Then the regression line must have an intercept with the x-axis. This intercept point would be the optimum allele number that maximises LRS. Note that each sibship is a statistical unit providing one pair of data points each. The regression analysis is done with $N=6$ independent sibships, any pseudoreplication are avoided (Hurlbert 1984; Milinski 1997).

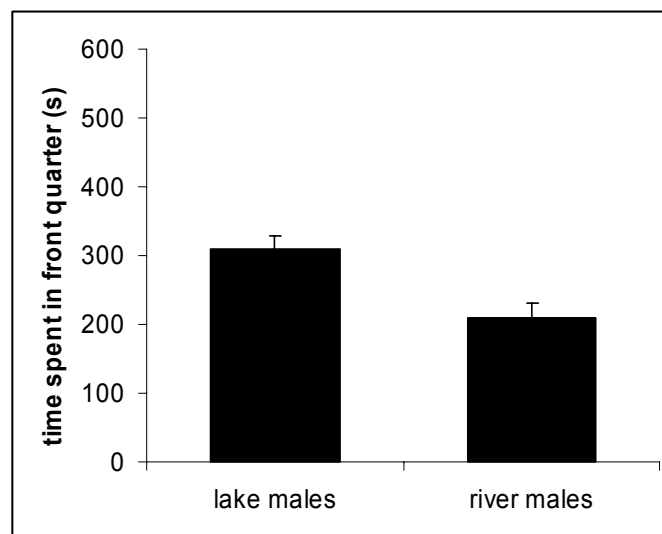
Supplementary Fig. 4: Fictitious example of LRS of two genotypes of each of three sibships. See text for further details.



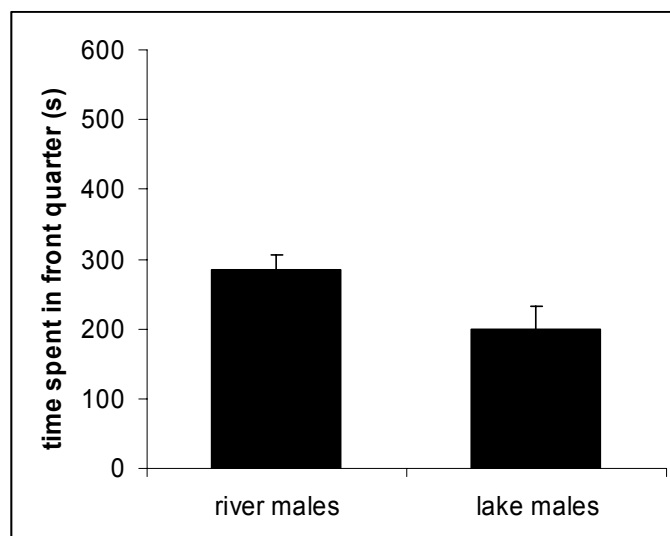
Appendix III

This part contains supplementary information of Chapter V. I reported in the main text of this chapter that in a flow channel experiment females significantly preferred more often the side of the water coming from the male originating for the same habitat. The supplementary figure (1 a) reports the preference for females originating from lake, while figure (1 b) reports preference of females originating from the river habitat.

Supplementary Fig. 1 a: Mean time + SE lake females spent in front quarter close to male water inflow (t-test, $t = 3.602$, d.f. = 18, $p < 0.005$. Binomial test, $p = 0.01074$).



Supplementary Fig. 1 b: Mean time + SE river females spent in front quarter close to male water inflow (t-test, $t = 2.126$, d.f. = 18, $p < 0.05$. Binomial test, $p = 0.05$).



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.

Außerdem versichere ich, dass ich bisher noch kein anderes Promotionsverfahren begonnen habe.

Teile dieser Dissertation wurden bereits veröffentlicht oder zur Veröffentlichung eingereicht. Mein Beitrag sowie der Beitrag meiner Co-Autoren ist am Anfang jedes Kapitels und in Tabelle 1 detailliert aufgeführt.

Plön, den 22. Oktober 2008.

Christophe Eizaguirre

