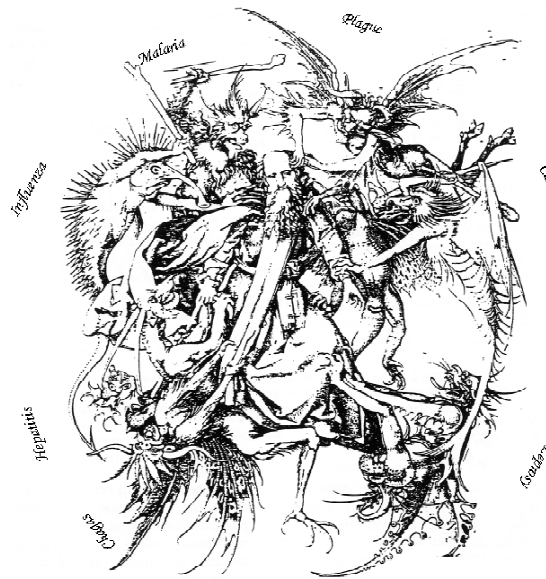


The role of parasite diversity in the life of three-spined sticklebacks



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Summary

Parasite infections containing genetically different parasites of the same species are widespread in nature. The genetic diversity is predicted to influence parasite load, the evolution of virulence and host defence mechanisms. According to kin-selection theory, competition is stronger between individuals of different genotypes than between genetically identical individuals. This leads to a lower parasite load in a diverse infection compared to a uniform infection. I studied the importance of genetically diverse infections for three-spined sticklebacks (*Gasterosteus aculeatus*) parasitised by the trematode *Diplostomum pseudospathaceum*. This parasite species has a complex life cycle comprising (a) a water snail as a first intermediate host, where asexual reproduction occurs, (b) a fish such as the three-spined stickleback as a second intermediate host, where growth takes place, and (c) a final host, a fish eating bird, where sexual reproduction takes place.

Using newly developed microsatellite markers (chapter 1), I showed that the diversity of the infection increases from the first intermediate host (water snail) to the second intermediate host (three-spined stickleback) in wild populations. This increase is so strong that almost every parasite individual infecting one stickleback host belongs to a different genotype (chapter 2). In experimental infections, I then demonstrated that different genotypes infecting one host do indeed suppress each other as predicted by kin-selection theory, leading to a lower total parasite load in a mixed genotype infection compared to single genotype infections (chapter 3). Besides competitive suppression, also a dominance effect can lead to a lower total parasite load: Increasing diversity increases the probability that a dominant genotype able to displace the co-infecting genotypes is included, which on the other hand causes itself only a low parasite load. To demonstrate that the reduced parasite load is indeed caused by competitive suppression rather than a dominance effect, I

used for the first time in parasitology a method originally developed for plant species diversity experiments, that allows distinguishing between competitive suppression and a dominance effect. As drug treatment success and infection development critically depend on the mechanism at work, I propose here the importance to distinguish between competitive suppression and the dominance effect.

Due to the omnipresence of parasites, their impressive diversity, both within and between species, and the large fitness costs they impose on their hosts, hosts developed a huge variety of defence mechanisms. The immune system of the host plays a central role for defence and its ability to recognise a parasite is often a prerequisite for a successful defence. In vertebrates, the immune system is broadly divided in innate and adaptive immune system. The innate immune system is based on the recognition of conserved molecular patterns. The defence is immediately ready, but is thought to be rather unspecific (i.e., it does not differentiate between different genotypes). In contrast, the adaptive immune system needs several days to become fully mounted and is highly specific (i.e., it does differentiate between different genotypes). A specific defence is needed for genotype-specific host-parasite interactions, a basic assumption of the Red Queen hypothesis. In such interactions, some parasite genotypes are better in infecting the host than others and the infection success depends on the host genotype. In the case of the three-spined stickleback and *D. pseudospathaceum*, I showed that genotype-specific interactions arise immediately (chapter 4). This rules out specific defence mechanisms of the adaptive immune system, as they need several days to get ready. Thus, speed and specificity are not necessarily mutually exclusive.

On a broader scale, parasite species diversity is besides genotypic diversity also an important contributor to total parasite diversity. I examined defence mechanisms against a diverse parasite community at the species level in the wild. MHC genes play a central role for the activation of the adaptive immune system. However, MHC genes did not significantly influence

parasite load when fish were experimentally exposed to the natural parasite community (chapter 5). In contrast, genomic background explained a significant percentage of the variation in parasite load, suggesting the importance of other defence mechanisms besides the MHC dependent adaptive immune system.

Vaccination often alters the genetic composition of an infection. The possible consequences of such drug treatment effects on disease severity are only beginning to be investigated. Advances in predicting how the effect of drug control on genetic diversity influences total parasite load is crucial for many important diseases. Further, understanding more about host defence mechanisms helps explaining how organisms manage to survive in a world full of parasites.

Zusammenfassung

Infektionen mit mehreren genetisch unterschiedlichen Parasiten der gleichen Art sind eine häufige Erscheinung in der Natur. Theoretische Modelle besagen, dass diese genetische Vielfalt die Parasitenanzahl, die Evolution der Virulenz und die Abwehrmechanismen der Wirte beeinflusst. Der Verwandtenselektionstheorie zufolge, ist die Konkurrenz zwischen genetisch unterschiedlichen Individuen der gleichen Art stärker als zwischen Individuen mit dem gleichen Genotyp. Dies würde dazu führen, dass die Parasitenanzahl in einer genetisch vielfältigen Infektion im Vergleich zu einer genetisch einheitlichen Infektion erniedrigt ist. Ich habe die Bedeutsamkeit genetisch vielfältiger Infektionen anhand des dreistachligen Stichlings (*Gasterosteus aculeatus*) und eines seiner Parasiten, dem Trematoden *Diplostomum pseudospathaceum*, untersucht. Dieser Parasit besitzt einen komplexen Lebenszyklus: Im ersten Zwischenwirt, einer Wasserschnecke, findet die asexuelle Fortpflanzung statt, im zweiten Zwischenwirt, einem Fisch, wie z. B. dem dreistachligen Stichling, wächst der Parasit, und im Endwirt, einem fischfressenden Vogel, findet die sexuelle Fortpflanzung statt.

Mit Hilfe von neuentwickelten Mikrosatelliten (Kapitel 1) habe ich gezeigt, dass die genetische Vielfalt der Parasiteninfektion innerhalb eines Wirtes vom ersten Zwischenwirt (Wasserschnecke) zum zweiten Zwischenwirt (dreistachliger Stichling) im natürlichen Habitat stark zunimmt. Dies führt dazu, dass fast jedes Parasitenindividuum einer Infektion im dreistachligen Stichling einen anderen Genotypen besitzt (Kapitel 2). Wie durch die Verwandtenselektionstheorie vorhergesagt, haben experimentelle Infektionen gezeigt, dass verschiedene Genotypen sich tatsächlich gegenseitig unterdrücken. Dies führt zu einer Erniedrigung der Parasitenanzahl in einer genetisch vielfältigen Infektion im Vergleich zu einer genetisch einheitlichen Infektion (Kapitel 3). Um nachzuweisen, dass es sich tatsächlich um

Unterdrückung handelt, habe ich eine Methode angewandt, die ursprünglich für Pflanzendiversitätsstudien entwickelt wurde. Diese Methode ermöglicht die Unterscheidung zwischen einem Unterdrückungs- und einem Dominanzeffekt. Auch ein Dominanzeffekt kann zu einer Erniedrigung der Parasitenanzahl führen. Mit der Erhöhung der genetischen Vielfalt erhöht sich auch die Wahrscheinlichkeit, dass ein dominanter Genotyp eingebunden wird, der zwar die anderen Genotypen verdrängen kann, selbst aber nur eine geringe Parasitenbelastung verursacht. Da sowohl die Medikamentenbehandlung als auch der Verlauf einer Infektion in hohem Masse davon abhängt, welcher dieser zwei Effekte vornehmlich die Parasitenbelastung bestimmt, ist die Unterscheidung zwischen Unterdrückungseffekt und Dominanzeffekt von zentraler Bedeutung.

Durch die immense Parasitenvielfalt, ihrer allgegenwärtigen Präsenz und die hohen Fitnesskosten, die sie verursachen, haben die Wirte eine grosse Vielfalt an Abwehrmechanismen entwickelt. Grundlage für eine erfolgreiche Bekämpfung ist oft die Erkennung des Parasiten. Wirbeltiere besitzen eine angeborene Immunität, die einen eher unspezifischen Erkennungsmechanismus besitzt (d.h., sie unterscheidet nicht zwischen verschiedenen Genotypen), aber sehr schnell auf eine Infektion reagieren kann. Andererseits besitzen Wirbeltiere eine induzierte Immunität, die zwar hochspezifisch ist (d.h., sie kann auch zwischen verschiedenen Genotypen unterscheiden), aber einige Tage braucht bis sie vollständig aktiviert ist. Die spezifische Bekämpfung von Parasiten ist die Basis für genotyp-spezifische Wirt-Parasiten-Interaktionen, eine zentrale Annahme der Red-Queen-Theorie. Aufgrund solcher spezifischer genetischer Interaktionen sind einige Genotypen in der Lage, einen Wirt besser zu infizieren als andere und der Infektionserfolg ändert sich von Wirtsgenotyp zu Wirtsgenotyp. Anhand des dreistacheligen Stichlings und seinem Parasiten *D. pseudopathaceum* habe ich gezeigt, dass genotyp-spezifische Wirt-Parasiten-Interaktionen innerhalb von 24h erfolgen können (Kapitel 4). Dabei können spezifische Mechanismen der induzierten Immunität ausgeschlossen werden, da diese einige Tage

brauchen bis sie verfügbar sind. Folglich schliessen sich eine schnelle und trotzdem spezifische Abwehr nicht notwendigerweise gegenseitig aus.

Neben der genotypischen Vielfalt spielt auch die Artenvielfalt eine grosse Rolle. Ich habe die Bedeutung von Abwehrmechanismen gegen eine vielfältige Parasitenartengemeinschaft im natürlichen Habitat untersucht. Dabei spielen MHC-Gene eine wichtige Rolle, um das induzierte Immunsystem zu aktivieren. In einem Experiment, in dem ich Fische mit unterschiedlichen MHC-Genen der natürlichen Parasitenfauna ausgesetzt habe, habe ich jedoch keinen Einfluss von MHC-Genen auf den Parasitenbefall gefunden (Kapitel 5). Der genetische Hintergrund der Fische dagegen hatte einen starken Einfluss auf den Parasitenbefall. Dies deutet darauf hin, dass neben dem MHC-abhängigen induzierten Immunsystem auch andere Abwehrmechanismen eine wichtige Rolle spielen.

Die Behandlung mit Medikamenten verändert oft die Zusammensetzung und Vielfalt der Genotypen einer Infektion. Daher ist die Voraussage, ob und wie die veränderte genotypische Vielfalt sich auf die Parasitenbelastung auswirkt, von zentraler Bedeutung für viele bedeutende Krankheiten. Ausserdem helfen Erkenntnisse über die verschiedenen Abwehrmechanismen eines Wirtes gegen Parasiten zu verstehen, wie Organismen es schaffen trotz der allgegenwärtigen Präsenz von Parasiten zu überleben.

Introduction

1. The role of parasite diversity

Parasites are one of the major forces driving the evolution of organisms (Hamilton 1980; Ebert 1994). Reasons for this prominent role are the considerable fitness reductions they can cause in their hosts and their omnipresent distribution. The diversity of parasites is breathtakingly large (figure 1). It has been suggested that half of the existing species are parasites (May 1988). Diversity within species is suggested to be even more important, as the major part of the total diversity is contributed by differences between genotypes of the same species (Harper 1977). Parasites are, following the definition of Begon *et al.* (1996), organisms that live in a close association with another organism (the host). In such an association, parasites exploit the resources of the host and cause a fitness reduction to the host.

Whether or not an organism is infected by a parasite population with low or high genotypic diversity plays a central role for the evolution of virulence (Nowak *et al.* 1991; Frank 1996). Virulence is the amount of damage a parasite causes to its host. A parasite that relies on the survival of its host to reproduce should optimise the level of virulence to increase the reproduction rate. Over-exploiting the host can be fatal if the host dies before the parasite was able to successfully reproduce. On the other hand, some level of exploitation is necessary for the parasite to finish development or for reproduction. This cost-benefit balance is disturbed, when different parasite genotypes infect the same host. The reason is that in the competitive situation, parasites, which exploit the host more rapidly to increase their reproduction rate, are favoured. A higher exploitation rate may then lead to a greater damage to the host and hence to a higher virulence. Indeed, several

models predict that in mixed-genotype infections virulence evolves to higher levels (Frank 1996; Ebert 1998; Nowak & May 1994; Brown *et al.* 2002).

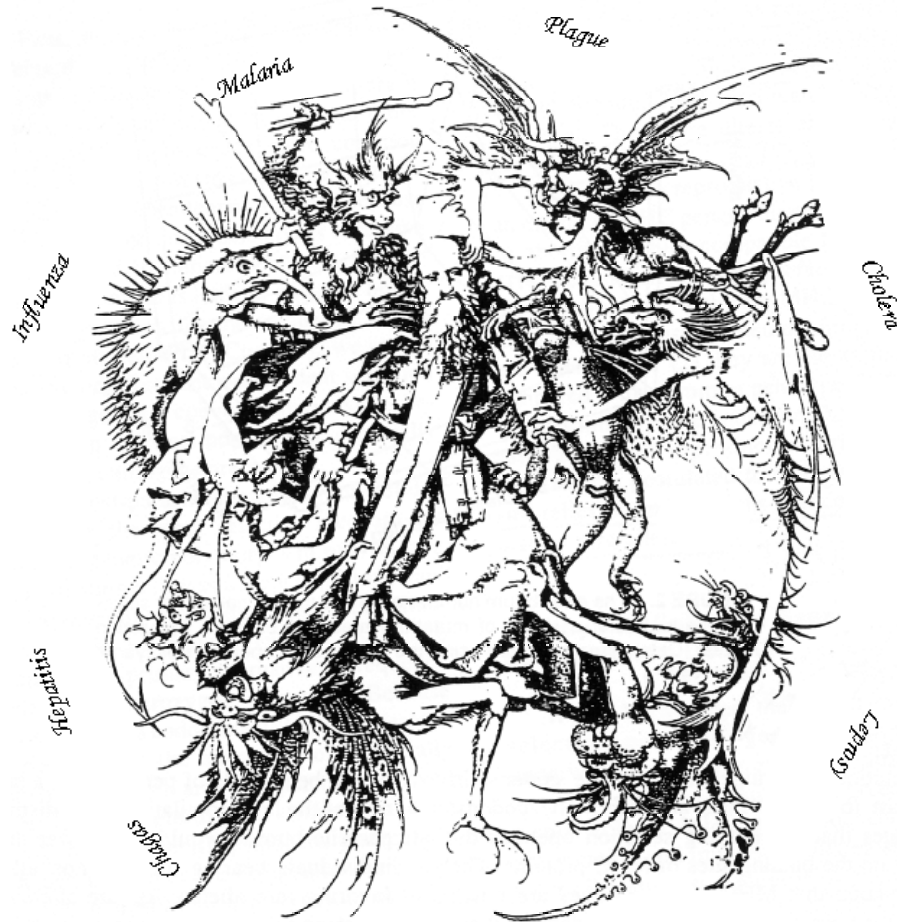


Figure 1: The sage threatened by numerous diseases (modified from a drawing from Apanius *et al.* 1997 and with friendly permission of the journal Critical Reviews in Immunology, Begell House, Inc.).

However, an enhanced exploitation of the host is not the only way a parasite genotype can outcompete co-infecting genotypes. Suppressing the growth of co-infecting genotypes is another way (Chao *et al.* 2000). A suppression of competitors could occur with aid of chemical substances that harm only co-infecting genotypes but not the producing genotype itself, as shown in vitro for viral and bacterial pathogens (Hart & Cloyd 1990; Riley & Gordon 1999). If this suppression ability entails a cost for the own growth rate, virulence actually evolves to lower levels in mixed-genotype infections.

The genetic diversity of an infection also plays a central role for disease severity (Read & Taylor 2001). According to kin selection theory (Hamilton 1963), competition is stronger between individuals of different genotypes than between genetically identical individuals (Giron *et al.* 2004). This leads to a lower parasite load in a diverse infection compared to a uniform infection. Indeed, such a competitive suppression has been repeatedly found. In studies using molecular markers to distinguish the different genotypes also in mixed infections, in two-clone mixed infections, one clone was suppressed compared to monoclonal infections, while leaving the second clone unaffected (Taylor *et al.* 1997; de Roode *et al.* 2004; Gower & Webster 2005). Two seminal studies then showed for several combinations of mixed infections of two genotypes that the parasite infection rate or load was in general reduced in the mixed infections compared to the uniform infection (Wille *et al.* 2002; de Roode *et al.* 2005). This suggests that genotypic diversity has a negative effect on total parasite load.

The diversity of the parasite population during infection has also important implications for medical disease control (Read & Taylor 2001). Vaccines and other medical applications most probably alter the genetic diversity of infections. The consequences of such interventions for public health is far from being understood. The release of drug-resistant strains or of highly virulent strains is just one scenario. Another is that disease propagation may be slowed down or enhanced, depending on how genetic diversity influences transmission.

2. Regional diversity

Parasite diversity has multiple levels. Aside from within-host population diversity, it can also be partitioned among contrasting host locations. At a regional scale different habitat types may harbour different parasite species (Kalbe *et al.* 2002), and presumably, genotypes. Local adaptation of the host may lead to a better defence against the local parasite species than against foreign parasite species. Local adaptation means that the fitness is higher in the home habitat than in a foreign habitat (Kaltz & Shykoff 1998). However, also the opposite can occur, when parasites are better in infecting the local hosts. Local adaptation of organisms is frequently found (Ebert 1994; Lively 1999) and both the host and the parasite have been reported to be locally adapted. In a meta-analysis, Lajeunesse & Forbes (2002) suggested that parasites specialised on a single host are more likely to be locally adapted. In contrast, parasites that can infect several host species were, more likely to be maladapted and thus showing host local adaptation.

3. The role of biodiversity for ecosystem functioning

The central role of biological diversity for understanding intra and interspecific dynamics is not restricted to host-parasite interactions. The importance of biodiversity for ecosystem functioning has become more and more a central topic in public interest, as human activity often leads to loss of species diversity (Loreau *et al.* 2001). A main topic in the biodiversity area concerns the question whether an increase in biodiversity also increases productivity (Tilman 1999; figure 2). Numerous studies have manipulated species diversity in experimental plant communities to answer this question. There, the plant productivity was compared between monocultures and plant communities containing an increasing number of plants species. In general, these studies found an increase total biomass production with increasing

species number (e.g. Tilman *et al.* 1996; Hector *et al.* 1999; Roscher *et al.* 2005). Potential mechanisms resulting in positive effects of biodiversity are either niche differentiation (i.e. differences in resource use) or facilitation (positive interactions) between species (Loreau & Hector 2001). A prominent example of facilitation is the beneficial effect of legumes on non-leguminose plants. The latter can only access mineral nitrogen sources in the soil and profits from the presence of legumes, which can bind atmospheric nitrogen.



Figure 2: Examples of a species poor rapeseed field and a species rich rainforest (photos with friendly permission from Josef Foos and Tim Wellhausen).

How biodiversity may influence the stability of ecosystems is also of central interest in ecology. An increased biodiversity may enhance the resistance to disturbance, the rate of recovery after disturbance, and temporal stability (Tilman 1999). This is especially important, as human impact on the climate is predicted to lead to an increasing variability of the environment, which can entail heat waves or higher risk of flooding. Although very controversially discussed, Tilman (1999) concluded that diversity indeed stabilizes community and ecosystem processes.

Plant community experiments focus mainly on the role of diversity for ecosystem functioning. On the other hand, in parasite infections the focus lies more on the importance of diversity for disease severity and the evolution of

virulence. However, many of the theoretical models and assumptions developed for plant community experiments, can also be used for parasite infections and vice versa. The reason is that plant communities and parasite infections overlap in important characteristics. First, parasites infecting one host also share like plant communities one common resource pool. Second, genotypic diverse infections are widespread (Read & Taylor 2001), allowing the different genotypes to interact similar as in plant communities. Third, genotypic diversity, mostly studied in the field of parasitology, can be analysed analogous to species diversity, which is mostly studied in plant communities (Vellend & Gerber 2005). These parallels open a new field of applications to both fields, leading to promising interactive applications.

4. Host defences against parasites

Given the omnipresent treat from parasites, it is not surprising that hosts evolved a great diversity of defence mechanisms against parasites. Defence mechanisms against parasites can entail behavioural avoidance of parasites or structural defence like a thick outer-layer. A central defence mechanism is the immune system. The vertebrate immune system can be subdivided into the innate and adaptive immune system (Janeway *et al.* 1999). The innate immune system is a fast and effective first defence line against many pathogens, but is regarded as rather unspecific, as it depends on the recognition of conserved molecular patterns shared by large pathogen classes (Dixon & Stet 2001).

In contrast, the adaptive immune system is thought to be highly specific. The adaptive immune system is based on recognition of small protein fractions (peptides) and recognition may vary between different parasite clones and depend on the host genotype (Frank 2002). As only few receptors of immune cells can recognise each particular antigen, clonal expansion is necessary to build up an effective adaptive immune defence (Roitt *et al.*

1989). This induction process comes at the cost of a time delay of at least three to five days (Janeway & Medzhitov 2002). The adaptive immune defence achieves its extraordinary specificity by somatic rearrangement of genes that encode antigen receptors, i.e. T cell receptors and antibodies (Fearon & Locksley 1996). For the activation of an adaptive immune response, genes of the MHC play a key role (figure 3).



Figure 3: Structure of an MHC molecule class II complex. A bound peptide is displayed in red (picture with friendly permission from Song Tan, http://golan.sfbmb.psu.edu/faculty/tan/-lab/gallery/mhc_ribbon1.jpg).

MHC proteins play such a central role for the adaptive immune response because they initiate the proliferation of effective T-cell clones when presenting peptides derived from pathogens to T cell receptors. Note that self-peptides are also continually presented by MHC molecules, but they do not lead to an immune response (except in certain autoimmune disease) because auto reactive T-cell clones have been eliminated during ontogeny of the immune system.

The MHC can be subdivided in two main classes (Roitt *et al.* 1989). MHC class I encoded proteins mainly present peptides of intracellular parasites, such as many viral or bacterial pathogens (Hughes & Yeager 1998). This normally results in destruction of the infected cell by cytotoxic T cells. In contrast, MHC class II encoded proteins mainly present peptides derived from extracellular parasites, such as extracellular bacteria and macroparasites, to T helper cells (Penn & Potts 1999). This may initiate a complex immune reaction cascade that can finally lead to the production of specific antibodies.

Although invertebrates have long been thought to lack an adaptive immune system, there is increasing evidence for a radically opposite view. The immune response can be induced (Moret & Siva-Jothy 2003) and specific memory exists (Kurtz & Franz 2003). Thus, recent studies indicate that invertebrates, thought to rely only on innate immunity, possess a specific defence and memory (reviewed in Kurtz 2005).

5. Study system

5.1 *The host species three-spined stickleback*

Three-spine sticklebacks *Gasterosteus aculeatus* are widely distributed north temperate fish. Fish from presumably marine populations recolonised freshwater habitats after the last glaciation 12 000 years ago (Reusch *et al.* 2001b). Since then, three-spine sticklebacks showed a remarkable ability to diverge into different ecotypes. The differences comprise morphological traits, coloration and behaviour. Using genetic markers, Reusch *et al.* (2001b) showed that three-spined sticklebacks living in river habitats were also genetically divergent from fish living in lake habitats. After the establishment of a method to measure MHC diversity (Binz *et al.* 2001), it was shown that river and lake sticklebacks have almost completely divergent MHC genotypes, as

revealed by sequence comparison (Reusch T.B.H., Wegner K.M., Kalbe M. and Stam W.T., unpublished data).

5.2 The parasite diversity

Three-spined sticklebacks host an amazing diversity of different parasite species. Besides viruses, bacteria, fungi and protozoans, macroparasites are probably very important agents driving the evolution of sticklebacks (figure 4).

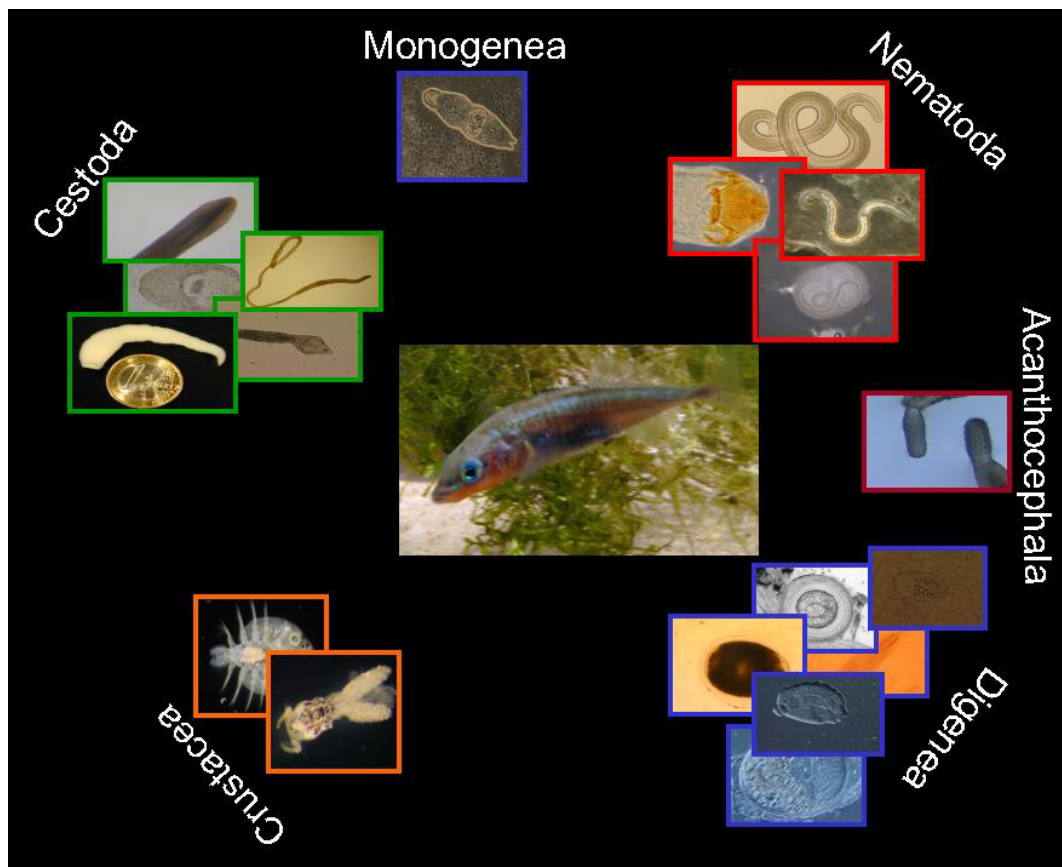


Figure 4: Parasite diversity of three-spined sticklebacks. Parasites species shown in this figure are by no means exhaustive, but were chosen to represent the different classes (photos with friendly permission from Martin Kalbe).

We concentrated on macroparasites due the possibility to distinguish different species morphologically and due to the relative small numbers, which makes it easier to quantify the parasite load. Although only concentrating on macroparasites, the diversity is still large. So far, more than 30 species of parasites have been found in sticklebacks from river and lakes in northern Germany (Kalbe *et al.* 2002). The range includes species from the classes of Acanthocephala, Cestoda, Ciliata, Crustacea, Digenea, Monogenea and Nematoda.

5.3 The eye-fluke *Diplostomum pseudospathaceum*, a special parasite

To study the genetic diversity within one species, we chose the trematode parasite *Diplostomum pseudospathaceum*. The reason is that it has an asexual reproduction phase, an ideal prerequisite for experimental infection experiments, as it allows replication of infections with individuals with an identical genome. Further, this species has been shown to harm the infected three-spined sticklebacks by decreasing food uptake rate and increasing predation risk (Crowden & Broom, 1980).

As most digenean trematode species, *Diplostomum pseudospathaceum* has a complex life cycle that needs three different host species to complete development (figure 5). It uses the freshwater snail *Lymnaea stagnalis* L. as a first intermediate host, where asexual reproduction takes place. Free-swimming larvae (=cercariae) are released from the snail. Numbers of larvae released per day can reach impressive levels under laboratory conditions, ranging between 7000 and 37000 cercariae per day and snail and this high level of cercariae release can go on over more than two months (Karvonen *et al.* 2004). Free-swimming stages penetrate into the second intermediate host (fish), here the three-spined stickleback *Gasterosteus aculeatus* L. Once inside the fish, the parasite migrates within 24h through the body into the eye lens, where it is protected from the immune

system (Chappell *et al.* 1994). Within the eye-lens, growth takes place (fish stage: metacercariae). The definitive host, a fish eating bird such as a herring gull (*Larus argentatus*), eats the fish and sexual reproduction occurs in the bird's gut. The produced eggs are then passed out in the water, larvae hatch (miracidium) and penetrate the first intermediate host.

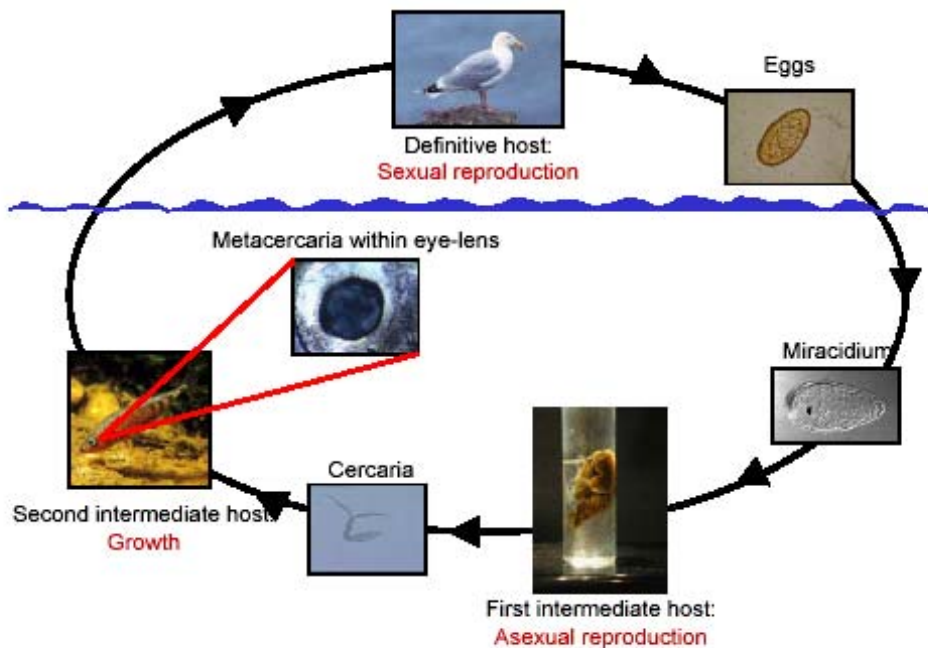


Figure 5: Life cycle of *D. pseudospathaceum*. Names above the pictures are the life-stage denomination. Description under the pictures describes the host and whether reproduction or growth occurs (photos with friendly permission from Martin Kalbe).

Thesis outline

Parasite diversity plays a central role for disease severity (virulence) and for the evolution of host defences. In this thesis, I studied these questions in the model organism, the three-spined stickleback, and its accompanying parasites. The outline gives an overview of my approach.

Chapter 1

Measuring genotypic diversity

Parasite diversity is composed of species diversity and within-species diversity (genotypic diversity). Parasite species diversity was determined morphologically in collaboration with M. Kalbe. For determining genotypic diversity, I developed genetic markers to distinguish the different individuals. I chose the trematode parasite *Diplostomum pseudospathaceum* for this purpose due to its asexual reproduction phase, which makes it an ideal organism for experimental infection. I developed a genetic assay using the polymorphism at five microsatellite loci. This allowed not only the distinction of individual parasite genotypes (=clones) but also general population genetic analyses of *Diplostomum* populations across their different life-cycle stages and host species.

Chapter 2

Measuring genotypic diversity in the wild

I used the microsatellite markers described in chapter 1 to estimate how widespread genetically diverse infections of *D. pseudospathaceum* are. I measured the genotypic diversity within single first and second intermediate hosts in the wild. Most individuals released by the first intermediate host

(snail) belonged to one clone. In contrast, the second intermediate host (fish) was infected with a diverse array of mostly unique parasite genotypes. This demonstrates that extensive clone mixing occurs from the first to the second intermediate host in natural populations. Thus, maintaining the second intermediate host in the parasite life cycle could be advantageous to reduce inbreeding in the final host.

Chapter 3

The negative effect of genotypic diversity of an infection

To test how this highly diverse parasite infections demonstrated in wild fish populations influence total parasite load, I experimentally infected three-spined sticklebacks with either a single clone or different clones of *D. pseudospathaceum*. Increased competition between unrelated genotypes may lead to a lower parasite load than expected from single-genotype infections. However, a dominance effect can also cause such a reduction. Increasing diversity also increases the probability to include a dominant genotype able to displace the co-infecting genotypes, but itself causing only a low parasite load (see summary). I used a method originally developed for plant diversity experiments to distinguish between competitive suppression and a dominance effect. The parasite load in mixed infections was lower than expected from single-clone infections. This reduction was caused by competitive suppression between co-infecting genotypes and not by a dominance effect.

Chapter 4

The fast and specific defence

To test whether three-spined stickleback defence is actually able to differentiate between different genotypes of *D. pseudospathaceum*, I studied the specificity of host defence mechanisms. In vertebrates, the immune defence against parasites is either fast and rather unspecific (innate immune

system) or needs several days to become fully mounted and is highly specific (adaptive immune system). I show that a combination of traits, usually exclusively attributed to only one of the two immune systems, is also possible, allowing a fast and at the same time highly specific defence. This was done by demonstrating genotype-specific host-parasite interactions that arose within 24h using a cross-infection experiment.

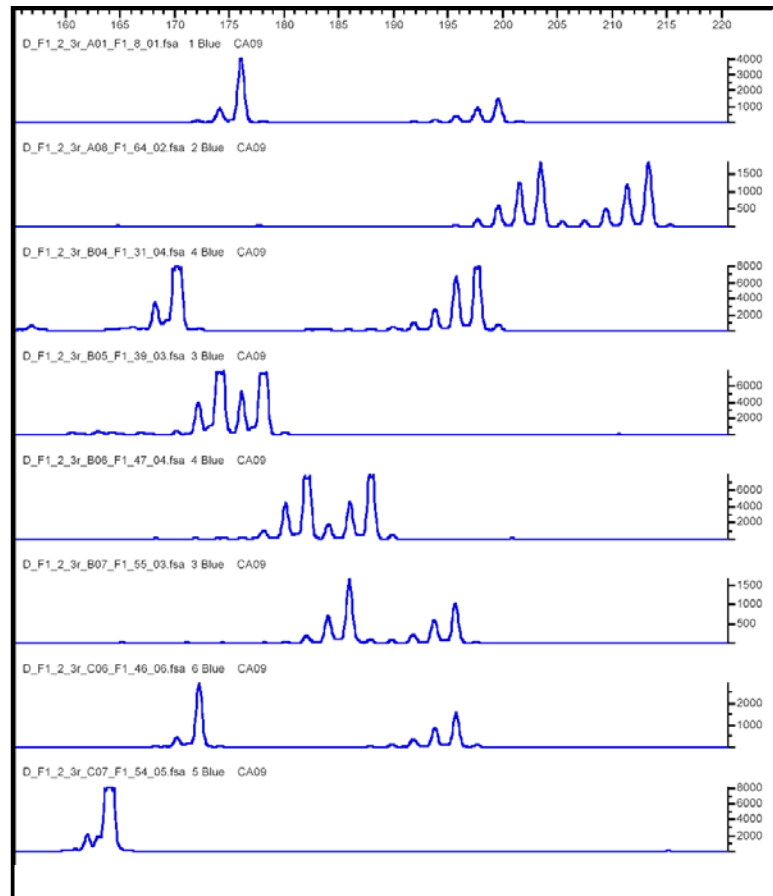
Chapter 5

The role of the adaptive immune system

Parasite diversity is not only restricted to within-species diversity as examined in the first four chapters. Differences between parasite species also contribute to the total parasite diversity. In the last chapter, I examine the importance of the immune system for defence against a diverse parasite community at the species level in the wild. MHC genes play a central role for the activation of the adaptive immune system. With a two-generation breeding design, I bred three-spined sticklebacks with different MHC complexes, but with the same randomised background genome. I exposed the fish in outdoor cages to the natural parasite communities. However, MHC genotype did not significantly influence parasite load, which was composed of various parasite species. In contrast, the genomic background explained a significant percentage of the variation in parasite load. This suggests the importance of defence mechanisms beyond the MHC region.

Chapter 1

Measuring genotypic diversity



Abstract

We present primers for five polymorphic microsatellite loci in the eye fluke *Diplostomum pseudospathaceum* (Trematoda), a widely distributed parasite with a complex life cycle used as a model for parasitology and fish immunology. The loci were identified using a GA/CT-enriched genomic library using subtractive hybridisation with magnetic particles. All five loci were highly polymorphic displaying 17 - 61 alleles and heterozygosities ranging from 0.53-0.92. We isolated populations of parasites within the first (snail) and second intermediate host (fish), and found small, but significant genetic differentiation ($F_{ST} = 0.012$) between the two life stages of the parasite.

Introduction

The eye fluke *Diplostomum pseudospathaceum* NIEWIADOMSKA 1984 is a widely distributed parasite in the eye lenses of freshwater fish. This trematode is a known cause of diseases and mortality both in commercial fish farming, but also in natural populations (Chappell *et al.* 1994). Therefore, it is widely used as a model for the epidemiology, ecology and physiology of digenean trematodes. The life cycle of *D. pseudospathaceum* involves snails (*Lymnaea stagnalis* or *L. palustris*) as first intermediate hosts and a wide range of fish species as second intermediate hosts. Infected fish are eaten by the parasite's definitive hosts, mainly gulls, where sexual reproduction takes place.

For parasite species with life stages within hosts, it is particularly useful to develop species-specific primers that do not amplify co-occurring host tissue (Binz *et al.* 2000). Here, we report the development of five species-specific primers for *D. pseudospathaceum*. Correct taxonomic determination is a critical issue within trematodes, and the genus *Diplostomum* in particular. The identification of *D. pseudospathaceum* was done under phase contrast microscopy (x 1000) of the cercariae, the second larval stage penetrating a fish, according to Niewiadomska & Kiseliene (1994).

Methods

For the genomic library, 100 parasites were isolated from the eye lenses of several experimentally infected sticklebacks (*Gasterosteus aculeatus*). DNA was extracted using the DNeasy tissue kit (Qiagen). For enrichment with GA/CT-motifs we used a hybridisation selection method (Kijas *et al.* 1994). We ligated short, self complementary oligonucleotides to the restriction site of the target DNA fragments (Edwards *et al.* 1996) after restriction of the total DNA with *Sau3A*. Double-strand linker molecules with a

5'-GATC overhang were produced by denaturing and snap-cooling the oligonucleotides linker-F (5'-TTGCTTACGCGTGGACTC-3') and linker-R (5'-GATCGAGTCCACGCGTAAGCAA-3'). We amplified 5 µl of the ligation product in a 50 µl-PCR containing 0.1 % BSA, 250 µM of each dNTP, 0.4 µM Primer (only linker-F), 1.5 mM MgCl₂, 1 x Promega PCR buffer (10 mM Tris-HCl, 50 mM HCl, 0.1 % Triton X-100) and 2.5 units of Taq DNA polymerase (Promega). The thermal profile consisted of an initial denaturation step of 94°C for 4 min, 28 cycles of 94°C for 30 sec, 60°C annealing for 1 min, 72°C extension for 2 min. The PCR-amplified DNA fragments that ranged from 400-750 base pairs in length were then subjected to hybridization with a (GA)₁₃-oligonucleotide probe that was labelled with dideoxy-guanin-triphosphate at the 3'-end to eliminate PCR-artifacts after the enrichment (Koblitzkova *et al.* 1998). The probe was 5'-bound via biotin-streptavidin to paramagnetic particles (Dynal, Kijas *et al.* 1994). Hybridisation reactions (100 µl) took place under gentle agitation at 60°C in 6xSSC (standard sodium citrate) including 0.1% SDS. The bead suspension was washed at 60°C with volumes of 500 µl SSC in triplicate with increasing stringency until 0.5xSSC. Initial tests revealed that the fraction containing the highest proportion of microsatellites would elute at 75°C with 0.2xSSC. The eluted microsatellite containing fraction was desalted and PCR amplified as above. The fresh PCR product was ligated and cloned into competent *E. coli* strains using the TOPO-TA cloning kit (Invitrogen) following the manufacturer's protocol. A high percentage of the bacterial plasmid clones contained DNA fragments with microsatellites (>60%).

One hundred-forty-four colonies were picked, and the plasmid DNA was extracted and sequenced with M13-primers using the Big-dye 3.1-sequencing kit (Applied Biosystems). Electrophoresis and sequence detection took place on an ABI 3100 automatic sequencer. Primer pairs were designed for 12 candidate loci using the software Primer3 (Rozen & Skaletzky 2000). To determine the microsatellite genotype of single parasites (cercariae from snails; or metacercariae from eye lenses of fish) the DNA was extracted with

the DNeasy tissue kit (Qiagen). Twenty- μ l PCR reactions contained 3 μ l DNA-extract, 0.1% BSA, 250 μ M dNTP, 1.5 mM MgCl₂, 1xPCR buffer, 2.5 units of Taq DNA polymerase (Promega), and 0.13 - 0.45 μ M of each of 2 primer pairs (Table 1-I). PCR started with 3 min at 94°, followed by n cycles (cf. Table 1-I) of 94°C for 1 min, 56°C for 1 min and 72°C for 1 min, followed by a final step of 72°C for 25 min. PCR reaction 'A' had a touchdown thermal profile for the first 10 cycles, starting at 61°C with a 0.5°C decrease for 10 cycles until 56°C was reached; for the remaining cycles and all other reaction profiles (i.e. 'B' and 'C') the annealing temperature was 56°C. PCR products were size separated and scored against an internal standard (Rox 350) on an ABI 3100. Fragment length analysis was done using the software GeneScan 3.5 (Applied Biosystems). Of the tested primer pairs, five yielded consistent and polymorphic amplification products.

	Primer Sequence (5'-3')	Repeat	Reac	C_{prim}	N_{cyc}	N	R	$H_{\text{in_snail}}$	$H_{\text{in_fish}}$	GenBank Accessio n
<i>Diplo06</i>	F Fam-TGCTTACATTGAAGGATGTTTCG R CGCCTTTTAATACGAACCTTG	(GA) _n	A	0.15	30	36	84-232	0.65	0.55	AJ629250
<i>Diplo08</i>	F Hex-GCGGTTAGATGGATGGATG R TGCTGTTTTGTTGCCTGAC	(GA) _n -(GGA) ₂ - (GA) ₁₀ -GG- (GA) ₁₀	B	0.45	34	61	84-244	0.53	0.61	AJ629251
<i>Diplo09</i>	F Fam-CGTCAAAGTGACCAAACCTCG R AAAGCGGATACAAGGAATGC	(GA) _n	C	0.13	30	41	164-258	0.92	0.87	AJ629252
<i>Diplo23</i>	F Hex-TTTCGAGTGTCTGTGTGCAA R AGAACAAATGCCGTTTTCAA	(GA) _n	A	0.15	30	17	86-140	0.67	0.73	AJ629253
<i>Diplo29</i>	F Hex-AGCTGCTTTGTTGTCCAATC R TGTTGTGTCGAACATTGCTTAG	(GA) _n	C	0.2	30	43	138-256	0.71	0.61	AJ629255

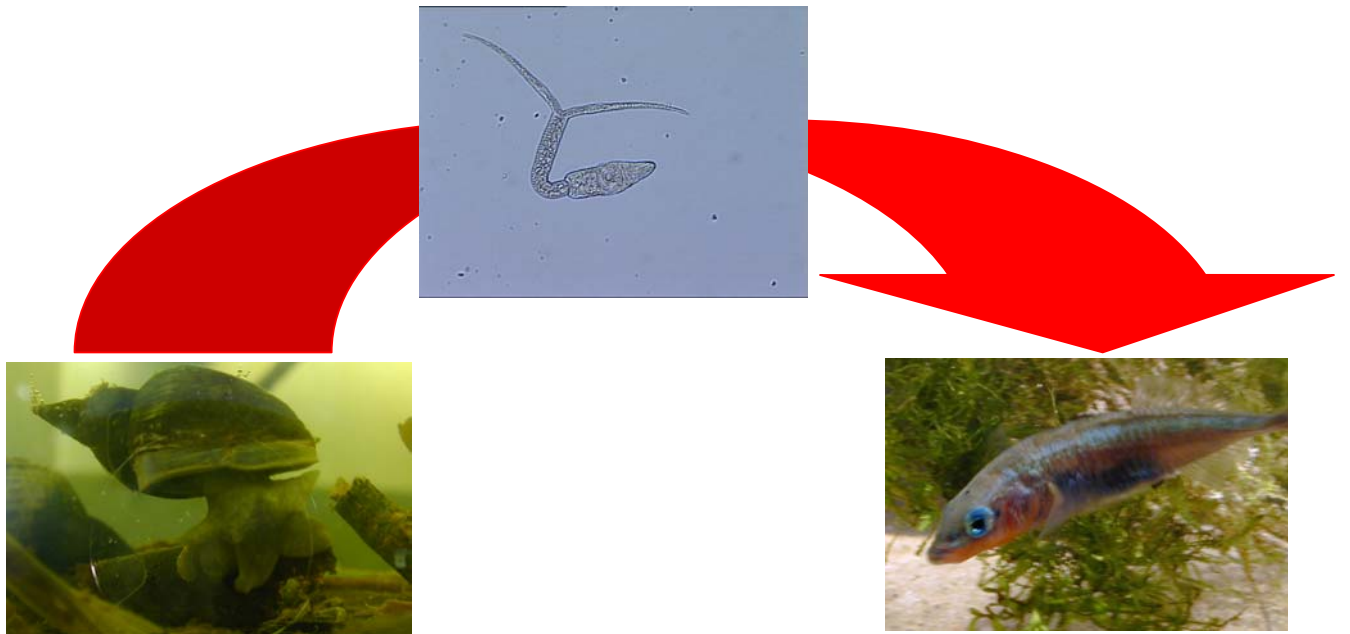
Table 1-I: Characterization of six microsatellite loci for *Diplostomum pseudospathaceum*. Reac = letter refers to the pooled PCR reaction, C_{prim} = primer concentration (μ M), N_{cyc} = number of PCR cycles, N = number of alleles observed, R = allele size range in base pairs, H = observed heterozygosity in parasites from snails ($N=50$), and from fish ($N=219$). See text for additional details on the thermocycler reaction profiles.

Results and Discussion

We subsequently genotyped a larger array of parasites derived from 10 snails (*L. stagnalis*, $N= 401$) and isolated from eye-lenses of 10 three-spined stickleback (*Gasterosteus aculeatus*, $N= 231$) from Kleiner Plöner See, Schleswig-Holstein, Germany. Since *D. pseudospathaceum* reproduces clonally within the snail, identical genotypes (=clones) were excluded from the subsequent analysis. Locus polymorphism was calculated using the software GENETIX (Belkhir *et al.* 1996-2002). Allele numbers and observed heterozygosities were high (Table 1-II). The locus *Diplo08* was particularly polymorphic. Here, amplification artifacts ('stutter bands') of genotypes with both alleles >160 bp (i.e. more than 40 dinucleotide repeat units) did not allow to distinguish with certainty between homo- and heterozygote individuals. This applied to 14% of genotypes of *Diplo08*, resulting in an underestimation of heterozygosity. In order to test for Hardy-Weinberg equilibrium we calculated F_{is} using GENETIX and found consistent positive deviation of F_{is} from zero across all loci in both, snail and fish stages of the parasite (all $P < 0.05$; $F_{is} = 0.07-0.51$). Linkage disequilibrium was tested using the permutation approach implemented in GENETIX (2000 permutations). We detected significant linkage in parasites from the snails only (cercariae) among the locus pairs *Diplo23* and 29, *Diplo08* and 09, and *Diplo09* and 29. These locus pairs were clearly unlinked in 219 metacercariae from three-spined stickleback ($P > 0.10$). All other locus pairs were in linkage equilibrium in both snail and fish stages of *D. pseudospathaceum* (all $P > 0.10$; Bonferroni-correction applied). We found significant genetic differentiation among the populations in the first and second intermediate host ($F_{ST} = 0.012$, $P < 0.01$). Because of their high polymorphism, the presented primers will be valuable for studying clonal structure, population structure and mating system in this important fish parasite.

Chapter 2

**Measuring genotypic diversity
in the wild**



Abstract

How complex life cycles of parasites are maintained is still a fascinating and unresolved topic. Complex life cycles using multiple hosts, asexual and sexual reproduction and free-living stages are widespread in parasitic helminths. For such life cycles, we propose here that maintaining a second intermediate host in the life cycle can be advantageous to increase the intermixture of different clones and therefore decrease the risk of matings between genetically identical individuals in the definitive host. Using microsatellite markers, we show that clone mixing occurs from the first to the second intermediate host in natural populations of the eye-fluke *Diplostomum pseudospathaceum*. Most individuals released by the first intermediate host belonged to one clone. In contrast, the second intermediate host was infected with a diverse array of mostly unique parasite genotypes. The proposed advantage of increased clone intermixture may be a novel selection pressure favouring the maintenance of complex life cycles.

Introduction

Complex life cycles of parasites have fascinated biologists for long time (Gibson 1987; Dobson 1989; Rhode 1994; Combes 1997). Parasitic helminths comprise an astounding diversity of life cycles, using up to four hosts and a diverse array of transmission modes (Parker *et al.* 2003; Cribb *et al.* 2003). A widespread and large group of parasites with a complex life cycle are digenean trematodes. The wide majority of trematodes are using three hosts (Cribb *et al.* 2003). However, in several independent trematode families, individual species reduced their life cycle to two or even only one host (Combes 1997; Poulin & Cribb 2002). Since several independent reduction events occurred, strong selective forces seem to drive the evolution of reduced life cycles. A proposed advantage of omitting hosts is that the life cycle can be completed with less transmission steps and thus avoiding risks during the host switch (Poulin & Cribb 2002). Omitting hosts can also be advantageous if one host is temporarily very rare or if predation rate by the specific up-stream host during the trophic transmission is too low (Poulin & Cribb 2002).

Despite these advantages for omitting hosts, the majority of digenean trematodes have a life cycle with three hosts, suggesting strong counteracting forces that maintain hosts in the cycle. Such forces have been proposed to be higher growth and fecundity (Parker *et al.* 2003), an increased probability of finding a mating partner (Brown *et al.* 2001) and higher transmission rates (Morand *et al.* 1995; Choisy *et al.* 2003, Parker *et al.* 2003). These models were developed for parasites with sexual reproduction and trophic transmission (most nematodes and cestodes). Many parasite species, however, have in addition asexual multiplication in the first intermediate host and free-living stages actively penetrating the second intermediate host (most digenean trematodes, comprising several thousand species). This alternation between asexual and sexual reproduction may have important consequences for shaping the life cycle, which we explore in this paper.

Here we propose a different advantage for maintaining hosts in life cycles with asexual reproduction. Maintaining the second intermediate host increases clone intermixture and therefore decreases the risk of matings between genetically identical individuals in the definitive host, as follows. Rare infection events combined with asexual reproduction often leads to a first intermediate host infected by parasites all belonging to the same clone (Sire *et al.* 1999) or to one of only three clones (Minchella *et al.* 1995). The free-swimming larvae released from the first intermediate host are diluted rapidly in the water. The second intermediate host may then collect parasites each belonging to a different clone. The dilution effect can be compared to sperm movement in externally fertilizing animals, where sperm concentration decreases exponentially with increasing distance from the source (Denny 1988). Collecting parasites of different clones can work in two non-exclusive ways. First, the second intermediate host is very mobile so that clones can be collected from many different spots and from many different first intermediate hosts. This is a probable scenario for around half the trematode species using a vertebrate as second intermediate host (Cribb *et al.* 2003). Second, collection of different clones can occur through time, if changes in water currents or novel infections of first intermediate hosts lead to a continuous change in the clone pool. This mechanism may play a role for parasites using vertebrates as well as for less mobile hosts like molluscs or arthropods (Cribb *et al.* 2003). This dilution and collection mechanism leads to clone mixing, meaning here that individuals of one clone released by one first intermediate host do not end up in the same individual of the second intermediate host species.

If a second intermediate host is then eaten by the definitive host, it ingests parasites each belonging to a different clone. In contrast, if the second intermediate host is omitted and one first intermediate host is eaten directly by the definitive host, many parasites would belong to the same clone. Thus, the risk of matings between identical genotypes is increased with associated fitness costs of inbreeding (O'Brien *et al.* 1985; Charlesworth & Charlesworth

1987; Jimenez *et al.* 1994). The described reduction of the life cycle, where the parasite develops inside the first intermediate host into a life-stage able to infect directly the definitive host and where it awaits ingestion by the definitive host is not just a theoretical sketch. Although only rarely found, this special type of reduction is independently realised in four of the well over 100 trematode families (Poulin & Cribb 2002).

In this study, we tested whether clone mixing occurs for the eye-fluke parasite *Diplostomum pseudospathaceum* Niewiadomska from the first intermediate host (snail) to the second (fish). By directly genotyping single parasite individuals with microsatellite markers, this study is to our knowledge the first to directly test for clone mixing of parasites in a complex life cycle. We propose, that this mechanism may be a novel advantage for maintaining hosts in complex life cycles.

Methods

Life cycle of parasite

Eye-fluke parasites of the genus *Diplostomum* (Trematoda) are found worldwide in freshwater systems. *Diplostomum pseudospathaceum* uses the freshwater snail *Lymnaea stagnalis* L. as a first intermediate host, where asexual reproduction takes place. Free-swimming larvae (=cercariae) are released from the snail. Numbers of larvae released per day can reach impressive levels under laboratory conditions, ranging between 7000 and 37000 cercariae per day and snail. Such an abundant release of cercariae continued for more than two months (Karvonen *et al.* 2004). Free-swimming stages penetrate into the second intermediate host (fish), here the three-spined stickleback *Gasterosteus aculeatus* L. Once inside the fish, the parasite migrates into the eye lens of the fish, where growth takes place (fish stage: metacercariae). The definitive host, a gull, eats the fish and sexual reproduction occurs in the bird's gut. The produced eggs are then passed out in the water, larvae hatch and penetrate the first intermediate host. The

parasite harms both intermediate hosts. Fitness reduction occurs by castrating the snail and increasing predation risk of the fish (Crowden & Broom 1980). The life span of snails and fish are in the same range. *Lymnaea stagnalis* was reported to live up to 2.5 years (Väyrynen *et al.* 2000) and *G. aculeatus* a maximum of one to four years depending on the study site (Bell & Foster 1994). At our study site, sticklebacks are probably ecological annuals and seldom survive a second winter (unpublished observations).

In this study, we compared the genotypic (=clonal) diversity change from the first to the second intermediate hosts. Only this host transition is relevant for our hypothesis of clone mixing, because individuals living within a first intermediate host can end up in different second intermediate hosts. This mixing is not possible anymore from the second intermediate host to the definitive host, as due to the trophic transmission parasites living in one stickleback invariably end up in the same gull.

Sampling and clone typing

Snails and sticklebacks were caught in October 2003 in the lake Kleiner Plöner See (northern Germany). Snails were placed singly in a glass tube to check for released parasites. 41% of the 50 collected snails were infected and those released hundreds of cercariae within 12 hours. For each of ten infected snails, 47 released parasites were chosen randomly and singly genotyped. The test whether the clone composition of parasite populations within snails changed over time, all snails were kept singly in 2L aquaria at 18°C with 16h of light per day for two months. Water was exchanged every week and snails were fed once per week with green lettuce.

Of the ten randomly selected fish, all had eye-flukes. Six to 71 parasites were found per fish, of which all were singly genotyped.

We used five highly polymorphic microsatellites to assess the genotype of each parasite larva (Reusch *et al.* 2004, chapter 1). Clones were identified based on their multilocus microsatellite genotype. A new genotype was scored if at least one of the 10 analysed microsatellite alleles was different from the

previously analysed genotypes. Most genotypes differed at several alleles and only five genotypes differed only in one allele from a second genotype. As the markers were highly polymorph, the probability to find two individuals with the same genotype only due to segregation events was extremely low (mean probability for 10 randomly picked genotypes: $p < 10^{-10}$). Overall, 3% of the samples yielded no PCR-products and were excluded from further analysis.

Effect of sample size within snail parasites

Since we only analysed a small fraction of all cercariae one snail released, we tested how sample size affected the number of clones found using a re-sampling method. For every snail, we re-sampled the genotypes from one to 47 (maximum) of the cercariae without replacement. After 1000 runs, the mean number of genotypes detected was plotted against the number of cercariae sampled. A logarithmic curve was then fitted for each snail and the number of clones was extrapolated if 1000 cercariae would have been analysed, the estimated number one snail released within 12h.

Parasite clone diversity

We measured the diversity of the parasite population for every snail host and every fish host separately with the Shannon index, calculated with $H = -\sum p_i \ln(p_i)$, where p_i is the proportion of individuals in the i th clone for one host (Magurran 1988). As the number of individuals analysed differed between populations, we used rarefaction to standardise the diversity between populations (Gotelli & Entsminger 2001).

Calculation of F_{is} values

Inbreeding increases homozygosity (Charlesworth & Charlesworth 1987). To test for an excess of homozygotes, F_{is} values were calculated using the GENETIX program (Belkhir *et al.* 2002). Confidence intervals were calculated by bootstrapping the data set 1000 times. Null alleles are a problem for such analysis, as they increase the homozygote frequency. As we

amplified every microsatellite together with a second one, an estimation of the frequency of null alleles is possible. Every time one microsatellite of a pair amplified but the other not, a homozygote null allele was scored. The frequency of homozygote null alleles was calculated for every microsatellite and ranged from 0 to 0.053. For calculating F_{is} , only the microsatellite without any detected null alleles was used.

Results

Number of parasite clones within one host

The mean number of different clones a snail released was with 4.4 clearly lower than the average of 21.9 clones found within single fish (t-test: $t=-2.92$; d.f.=18; $P<0,001$). The number of clones ranged from one to nine clones per snail and from six to 67 clones per fish. The extrapolated number of different clones per snail if 1000 cercariae would have been sampled was 7.2 (s.e.=1.29). This is significantly higher than the number actually found (paired t-test: $t=4.58$; d.f.=9; $P<0,01$). However, the difference between the number of clones per snails and fish remained significant even when the extrapolated number of parasite clones released by one snail were used (t-test: $t=-2.40$; d.f.=18; $P<0,05$).

The majority of parasite clones were unique to one host. Only in one case, two individuals of the same clone were found in two different fish. In total, we found 44 different parasite clones for snails and 218 for fish.

Clone mixing

We clearly show that clone mixing occurs from the first to the second intermediate host. One clone clearly dominated the cercariae population released by a single snail (figure 1-II). On average over all 10 snails, the most abundant clone accounted for 65% (s.e.=6.94) of the parasite population one snail released with subsequent clones being less frequent. The second most abundant clone had already an average frequency of only 20% (s.e.=3.46).

The frequency decreased on average by a factor of 2.5 for each step down of the abundance hierarchy. In contrast, almost every parasite found within one fish belonged to a different clone (figure 1-II).

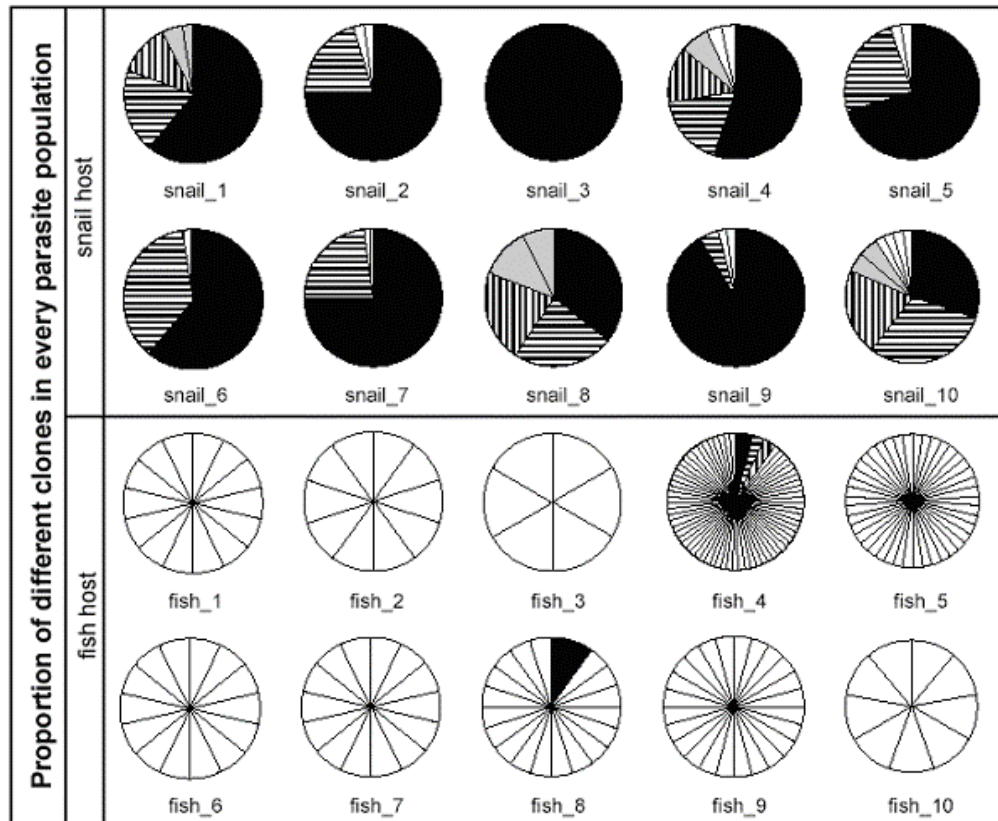


Figure 1-II: Proportion of different parasite clones in populations from single snails (cercariae) and fish (metacercariae). Black segments represent the proportion of the most abundant clone of one host, segments with horizontal lines the proportion of the second and segments with vertical lines of the third most abundant clone. All remaining clones are represented by a grey area. Each white segment represents a clone, where only one individual was found. Note that the same segment pattern in two hosts does not correspond to identical clones, but rather to both clones having the same relative abundance.

Only in three cases two individuals and once three individuals of the same clone were found within one fish (three of the four cases were within the same fish). Accordingly, the average diversity calculated with the Shannon index for the parasite population of snails was with 0.67 (s.e.=0.12) clearly much lower than for the parasite population of fish with 1.79 (s.e.=0.01) (Welch-ANOVA for means with unequal variances: $t=-9.11$; d.f.=9; $P<0.0001$). The effect size (= difference between diversity means/pooled s.d.) was 1.75, a large effect size by the standard suggested by Cohen (1988).

No parasite populations from snail or from fish had an excess of homozygotes. F_{is} values ranged from -0.43 to 0.33 . No value was significantly greater than zero and in eight populations even a deficit of homozygotes was detected ($P<0.05$).

The clone composition of parasite populations within snails changed only little over time. The cercariae of the five surviving snails were sampled again two month after the first analysis as above. In three snails, the most abundant clone found in the first round was still the most abundant and for two snails, it was still the second most abundant clone.

Discussion

Clone mixing...

Our data provided clear evidence for the postulated clone mixing from the first to the second intermediate host. While the snail released only few different clones and most of the snail stages belonged to one clone, the second intermediate host was infected with a diverse array of mostly unique parasite genotypes.

To be able to test for clone mixing, the genotype of single individuals must be assessed. Studies testing for the diversity change from the first to the second host have so far only pooled many parasites into one PCR product for each host. Therefore, it was not possible to find out how many individuals of the same clone were present within one host (for studies on *Plasmodium* see

Hill & Babiker 1995, Paul *et al.* 1995 and Arez *et al.* 2003 and on *Trypanosoma* Otieno *et al.* 1990, Hide *et al.* 2000 and Diosque *et al.* 2003). For *Schistosoma* parasites only, the diversity change between two hosts was examined based on data of single individuals. In these studies, however, the parasite population found in the first intermediate host was artificially grown by infecting laboratory reared mice in order to obtain enough parasite tissue for genetic analysis (Minchella *et al.* 1995; Barral *et al.* 1996; Sire *et al.* 1999; Curtis *et al.* 2002; Théron *et al.* 2004). As these passages are likely to alter the genetic composition of the parasite population (Wakelin *et al.* 2002), a direct comparison of the parasite diversity between the two hosts is not possible. To overcome these shortcomings, we genotyped single parasites directly after isolation from snails and fish. This study is, to the best of our knowledge, the first directly showing clone mixing of parasites in a complex life cycle.

We concentrated our study on one location, where we found a marked difference between parasite clone populations in snail and fish. Other sites may differ in many aspects and may also show a different degree of clone mixing. If for example fish have a strongly reduced mobility and snails are dispersed singly over wide distances, we would expect to find within fish clone frequencies that are more similar to those of snails. However, as soon as parasites of only two snails can intermix, we expect a higher diversity in fish than in snails as found in this study.

The parasite population of a snail was clearly dominated by a single clone. Why did we not find also in fish a frequency pattern with some clones being more frequent than others? The reason is that the number of different parasite clones existing in one habitat is extremely high. In our study, we found ≥ 250 different clones, but there are probably many more since our diversity estimate is far from reaching saturation. By intermixing this high number of different clones in the environment, the probability decreases to be infected by two individuals of the same clone, if only a low number of

parasites are sampled. In our study a fish accumulated in average only 22 parasites. It is important to note that each white circle segment in figure 1-II represents just one individual parasite. Any apparently even distribution of parasite clones within fish implies that most clones are only represented once while we cannot infer that parasites within fish are more evenly distributed than within snails.

...and the maintenance of complex life cycles

Here we propose that maintaining the second intermediate host in the cycle can be advantageous to increase clone mixing and therefore to decrease the risk of inbreeding in the definitive host. The reasoning is simple. If the definitive host eats directly one first intermediate host, it will be infected by parasites mainly belonging to one clone, in our study up to 100%. In contrast, if it eats one second intermediate host, it will be infected by parasites each belonging to a different clone. Clearly, the risk of matings between individuals of one clone and so the risk of inbreeding would be higher in the reduced life cycle. A main effect of inbreeding is to increase the frequency of homozygotes (Charlesworth & Charlesworth 1987). We did not detect an excess of homozygotes, indicating that inbreeding is not severe for the studied parasite. This is consistent with the proposed advantages of maintaining a second intermediate host in the life cycle to increase clone intermixture, which will ultimately reduce the risk of inbreeding.

The definitive host will probably eat many and not only one infected host. Therefore, in the reduced life cycle matings could also occur between parasites coming from different first intermediate hosts and so belonging to different clones. The increased risk in the reduced life cycle, however, prevails, because there would still be many individuals of the same clone in the same definitive host. In contrast, in the three-host cycle, the probability to encounter an individual of the same clone remains close to zero. If the definitive host would be infected with all 224 fish stages analysed in this study, 98% of the clones would be represented by one individual only. In addition,

there is a time effect. Parasites with the reduced life cycle would have to wait until an additional infected intermediate host is eaten to reduce the risk of mating with an individual of the same clone at least to some extent. However, waiting has probably costs, as adult parasites are not very long-lived and egg-production declines around three months after infection (Chappell *et al.* 1994). In contrast, parasites that use the second intermediate host can start mating right away without any risk of intra-clone matings.

Maintaining or losing intermediate hosts can be viewed as a balance between costs and benefits of complex life cycles. A reduction of the life cycle by losing the second intermediate host has arisen independently in several species of four trematode families (Poulin & Cribb 2002). This is an excellent possibility to test for forces maintaining complex life cycles by comparing several pairs of closely related species, one with the reduced life cycle and the other with the complete life cycle. If the above-mentioned mechanism of clone mixing works, we predict that species with a reduced life cycle have a reduced clone mixing and are more inbred compared to closely related species with complete life cycles. Some species of trematodes also reduced the cycle to a two-host cycle, but retained the free-swimming larval stage penetrating the definitive host (Poulin & Cribb 2002). In these species, clone mixing is still possible. Therefore, we expect that in this last case, inbreeding would not be as severe as in the species with two-host cycles without free-living stages. For those species with a two-host cycle keeping the free-swimming larvae, the proposed advantage of clone mixing can still work. In this case, clone mixing can be increased by maintaining the definitive host in the cycle. If the former definitive host is lost from the two-host cycle, asexual produced parasites may reproduce sexually within the only host before any dispersal takes place, as it is realised in several species of different trematode families (Poulin & Cribb 2002). There, the risk of inbreeding is evident.

Clearly, the proposed impact of clone mixing depends on how the life cycle reduction influences the transmission form. In this paper, we concentrated on the situation where the reduction leads to the loss of the free-

living stage and where clone mixing could be reduced. If the reduction leads, however, to the loss of the trophic transmission form and the free-living form is kept in the cycle, clone mixing is still possible. In this case, the proposed advantages of complex life cycles with trophic transmission (Morand *et al.* 1995; Brown *et al.* 2001; Choisy *et al.* 2003; Parker *et al.* 2003) may be more appropriate to explain the maintenance of the second intermediate host in the cycle.

Interactions with existing models

The proposed advantage of clone mixing in a multi-host cycle is additional and non-exclusive to existing models. Positive interactions can arise with already identified advantages of complex life cycles with trophic transmission. For example, Parker *et al.* (2003) proposed an increased growth and fecundity as an advantage of a complex life cycle. The parasite could combine those advantages and mix up the clones and at the same time increase growth and fecundity by keeping the second intermediate host in the cycle. An additional advantage of complex life cycles was proposed to be an increased transmission rate (Morand *et al.* 1995; Choisy *et al.* 2003; Parker *et al.* 2003). Maintaining the second intermediate host could increase transmission rate, if the definitive host feeds on many more second intermediate hosts than on first intermediate host. Again, the advantages can be combined allowing clone mixing and higher transmission rate by keeping the second intermediate host.

The mechanism of clone mixing is described here for parasites with asexual reproduction and free-living life stages. The general mechanism of genotype mixing may also be increased in a complex life cycle with exclusive sexual reproduction and trophic transmission compared to a simple life cycle. For example, in a two-host cycle with trophic transmission, the intermediate host may harbour closely related parasites, if parasite siblings infect the same intermediate host as they hatch in close spatial proximity. The definitive host may eat several first intermediate hosts and matings could occur between

parasites from different sibships. If a parasite leaves out the definitive host and reproduce sexually within the former intermediate host, the probability to mate with a sibling would be increased. The advantage of inbreeding avoidance through genotype mixing may therefore apply also in general for complex life cycles.

The origin of complex life cycles

It has long been assumed that the common ancestor of the digenean trematode was an endoparasite of molluscs that included later on the vertebrate host in the life cycle (Gibson 1987; Rhode 1994). A new phylogeny however, suggested a more parsimonious order with the parasite being first an endoparasite of vertebrates that included later on the mollusc host. Both transmission steps are thought to occur with free-living stages (Cribb *et al.* 2003; Littlewood *et al.* 1999). A likely explanation for the origin of complex life cycles is the following. If many free-living stages of the parasite were eaten by a predator, surviving the passage would clearly be a selective advantage and the predator may subsequently be integrated in the life cycle of the parasite (Smith-Trail 1980). In this case, clone mixing cannot explain the origin of complex life cycles, as asexual reproduction appeared after the complex cycle was already realised. However, genotype mixing in general could also have played a role in the origin of complex life cycles. With the inclusion of the second host, the cycle involves two steps with free-living stages and two steps could lead to an increased genotype mixing compared to one step.

In summary, we show that a mobile second intermediate host collects and mixes different genotypes released by intermediate hosts infected mainly by one clone. We propose a possible implication of this clone mixing for inbreeding avoidance as an advantage to maintain the second intermediate host in the life cycle.

Chapter 3

The negative effect of genotypic diversity of an infection



Abstract

The genetic diversity of an infection plays a central role for disease severity. Increased competition between unrelated parasite genotypes of the same species co-infecting one host may lead to a lower total parasite load in diverse infections than expected from single-genotype infections. However, a dominance effect can also cause such a reduction. Increasing diversity also increases the probability to include a dominant genotype able to displace the co-infecting genotypes, but itself causing only a low parasite load. In a novel approach, we used here an additive partition equation developed for plant biodiversity experiments, to study whether a single dominant genotype (selection effect) or a general competitive suppression between genotypes (complementarity effect) drives the mixed-infection outcome. We experimentally infected three-spine sticklebacks with either one, three or five clone(s) of a trematode parasite and distinguished clones in mixed infections with microsatellite markers. The parasite load in mixed infections was lower than expected from single-clone infections. We showed that this reduction was caused by competitive suppression between co-infecting genotypes and not by a dominance effect. As drug treatment success and infection development critically depend on the mechanism at work, we propose here the importance to distinguish between the complementarity and selection effect.

Introduction

Genetic diversity of infections critically affect disease outbreak and vaccination success (Nowak *et al.* 1991; Read & Taylor 2001). Infections containing different genotypes are widespread in nature (Woolhouse *et al.* 2002). Although positive interactions between co-infecting genotypes may occur, competitive interactions have received the greatest attention in theoretical models and experiments (Read & Taylor 2001). In experimental two-genotype-infections, competition was shown to suppress both co-infecting genotypes (Wille *et al.* 2002; de Roode *et al.* 2005) or only one of the two genotypes while leaving the second one unchanged (Taylor *et al.* 1997; de Roode *et al.* 2004; Gower & Webster 2005). In some cases, the initially suppressed genotype performed better in a late phase of the infection, indicating possible trade-offs (Taylor *et al.* 1997; de Roode *et al.* 2004). Such increased competition between unrelated genotypes co-infecting the same host is often proposed as a main force leading to a lower total parasite load in mixed infections than expected from single genotype infections (Chao *et al.* 2000).

However, a reduced total parasite load with increasing diversity can also be caused by a dominance effect. Increasing diversity also increases the probability to include a dominant genotype able to displace the co-infecting genotypes, but itself causing only a low parasite load. The total parasite load in the mixture then approaches the low parasite number of the dominant genotype in the single infection. This effect of increasing diversity is known from plant community experiments under the 'selection effect' (Loreau & Hector 2001). Competitive suppression on the other hand is described by the 'negative complementarity effect'. A negative complementarity effect occurs, when on average the different genotypes achieve a lower parasite load in mixed infections than expected from their respective single infections due to negative interactions between genotypes. Loreau & Hector (2001) presented a powerful tool to distinguish between the selection effect and

complementarity effect by means of an additive partitioning equation, which we adapted here for genotypic data. Both effects are potentially relevant (Loreau 2000). However, to measure whether in general genotypes suppress each other in a mixed infection, complementarity effect is the measure of choice.

The differentiation between the selection and complementarity effect plays a central role in mixed infections. For example, the development of an infection critically depends on the mechanism at work. With a strong negative selection effect, the dominant genotype displaces the other genotypes and the mixed infection becomes a single-genotype infection (Loreau 2000). With a negative complementarity effect, the infection remains diverse. Also, drug control can critically depend on the mechanism at work. In the case of a negative selection effect, drugs that were designed against subdominant genotypes do not greatly affect total number. However, eliminating the dominant genotype leads to clearance of the infection (if drugs are applied when the dominant genotype already displaced the other genotypes). In contrast in the case of a negative complementarity effect, the elimination of one genotype reduces total parasite number in the first instance. However, as growth rate of the remaining genotypes is increased due to the reduced competitive suppression, total parasite number can after a while actually exceed numbers in untreated hosts. Thus, we propose here the importance of the differentiation between the selection and complementarity effect to predict central questions for host-parasite interactions.

In a novel approach, we differentiated between competitive suppression (negative complementarity effect) and a selection effect for causing a reduction in total parasite load in genetically diverse infections, by applying the method developed by Loreau & Hector (2001) for biodiversity experiments in plant communities. To count the numbers of each genotype in the mixed infections we used microsatellite markers to distinguish between genotypes. Further we expand previous studies by including mixed infections containing more than three genotypes, by applying three levels of genotypic

diversity and by using five host lines in experimental infections of three-spine sticklebacks (*Gasterosteus aculeatus*) with the trematode parasite *Diplostomum pseudospathaceum*.

Methods

Study species

In the complex life cycle of the trematode parasite *Diplostomum pseudospathaceum*, sexual reproduction takes place in the gut of fish eating birds. Parasite eggs are shed into the water and hatching larvae infect water snails, where asexual reproduction occurs. Snails release free-swimming larvae, which infect fish, such as three-spined stickleback (*Gasterosteus aculeatus*). The parasite penetrates the skin of the fish and migrates within 24h through the body into the eye lens. There it awaits predation of the fish by birds to close the life cycle. Infected fish suffer from increased predation risk (Crowden & Broom 1980). Mixed-genotype infections are common in natural populations of three-spined sticklebacks and up to 67 different genotypes were found in a single fish (Rauch *et al.* 2005, chapter 2).

We established five clonal lines of the parasite *D. pseudospathaceum* by infecting single water snails (*Lymnaea stagnalis*) with single parasite larvae, as described in chapter 4. A successful parasite reproduced asexually within the snail, so that all parasite stages released from one snail belonged to one clone. Also, we established five unrelated fish families, descending from wild fish originating from the same lake system in northern Germany as the parasites and snails (for details on crossing procedure and rearing conditions see Rauch *et al.* 2006, chapter 5).

Infection design

We applied three diversity treatments with infections containing either one, three or five clones. In the one-clone infections, each fish got exposed to 150 parasites of one clone, in the three-clone infection to 50 parasites of each

included clone and in the five-clone infection to 30 parasites of each clone. All five clones were tested separately in one-clone infections. For the three-clone diversity treatment, we tested five different three-clone combinations. In a restricted random procedure, we randomly chose three clones from the five-clone pool. As soon as one clone was present three times in already chosen combinations, it could not be chosen anymore. This ensured that each clone was represented exactly three times. In the five-clone treatment, we mixed all five clones together. This resulted in five single-clone and six mixed-clone treatments. We exposed five fish from each of five families to each of these 11 clone treatments (except for one fish family, where only four fish were used due to small offspring number in that family).

Experimental infection

We placed the fish singly in 1l aquaria. To each fish, we added 150 parasites of the clone/clone mixture into the water not later than 12h after the parasites emerged from the snails. We randomly distributed the aquaria on tables in a climatic chamber (18°C, 16h light). We allowed the parasites to grow in size for one week. No reproduction occurs within the fish. We then killed the fish with an excess of methane sulphonate (MS 222), dissected and isolated the eye lens and counted the parasites within the undamaged eye lens.

To count the number of each clone in the mixed infections, we determined the clone identity of each parasite from all mixed infections. To differentiate between the clones, we used a microsatellite marker with a different length polymorphism for all five clones used. Length separation was done on an ABI 3100 automatic sequencer and each parasite got separately assigned its clone identity (for molecular methods see Reusch *et al.* 2004, chapter 1).

Selection and complementarity effect

We measured the change in parasite load in mixed-infections compared to single-infections with the net biodiversity effect, according to Loreau & Hector (2001) and adapted for genotypic data. The net biodiversity effect measures the deviation of the observed total parasite load in the mixture from the expected parasite load calculated from the single-clone infections. The expected value is for example in the five-clone infection the sum of the parasite loads in the five single-clone infections divided by five. A net biodiversity effect greater than zero means that the total parasite load in the mixture is greater than expected from the single-clone infections. An effect smaller than zero means that the parasite load in the mixture is smaller than expected.

The selection effect describes the situation where a clone with very high or very low parasite load in the single-clone infection dominates the mixture. This causes the total parasite load in the mixture to approach the parasite load of the dominating clone in the single-clone infection. This effect is measured by the covariance between the parasite load in the single-clone infection and the dominance in the mixture. The covariance increases (and the selection effect becomes greater than zero) if the dominant clone in the mixture also has a high parasite load in single infection and the subdominant clones a low parasite load. An effect smaller than zero means that dominant clones in the mixture have a low parasite load in single infections and the subdominant clones a high parasite load.

The complementarity effect measures whether in average the different clones achieve a higher or lower parasite number in the mixed infections than expected from its single-clone parasite load. For example in the three clone infections, if the number of parasites of one clone in the mixture is greater than one third of the parasite number in the single infection of that clone, it contributes a positive value to the average. A complementarity effect greater than zero means that the clones facilitate each other in mixed clone infections.

An effect smaller than zero means that the clones suffer from competitive suppression in mixed clone infections.

For each fish infected with a clone-mixture, we obtained one net biodiversity effect, selection effect and complementarity effect. We calculated the mean parasite number of the single-clone infections for each fish family and each clone separately. We compared parasite numbers from the mixed infections only with single-clone parasite loads coming from the same family. The sum of the selection effect and complementarity effect is the net biodiversity effect. All three effects can be positive or negative. The selection and complementarity effect can cancel out each other resulting in a zero net biodiversity effect.

Statistics

We tested for an influence of the three diversity treatments on total parasite number with a Welch Anova. This Anova allows variances being unequal, as untransformed and transformed data (log, square root, Box-Cox) did not achieve equality of variances (Levene test) needed for the standard Anova procedure. We controlled for family effect, by using for the Welch Anova the residuals gained from a one-way Anova with family as random factor.

We tested whether the net biodiversity effect, selection effect and complementarity effect were different from zero. We did this by including diversity treatment (either three-clone or five-clone mixtures) as fixed factor and fish family as random factor in a two-way Anova for each effect separately. The deviation of the intercept of this Anova from zero is the overall deviation of the effect tested (averaged over all factor levels). Net biodiversity effect was log transformed and selection effect Box-Cox transformed, so that all effects fulfilled the equality of variances (Levene test) and normality assumptions (Kolmogorov-Smirnov test).

Results

Total parasite number

Clone diversity significantly influenced total parasite number per fish (Welch Anova: $F_{2,92}=5.33$; $p=0.0065$). There was a significant decrease in parasite number from the single-clone infections to the five-clone infections, with the three-clone infections having an intermediate parasite load (figure 1-III). The variance was significantly different in the three treatments (Levene test: $F_{2,244}=12.78$; $p<0.0001$). The single-infection treatment had a higher variance than the two mixed infection treatments (multiple comparison with pair wise Levene tests and Bonferroni corrected for multiple testing).

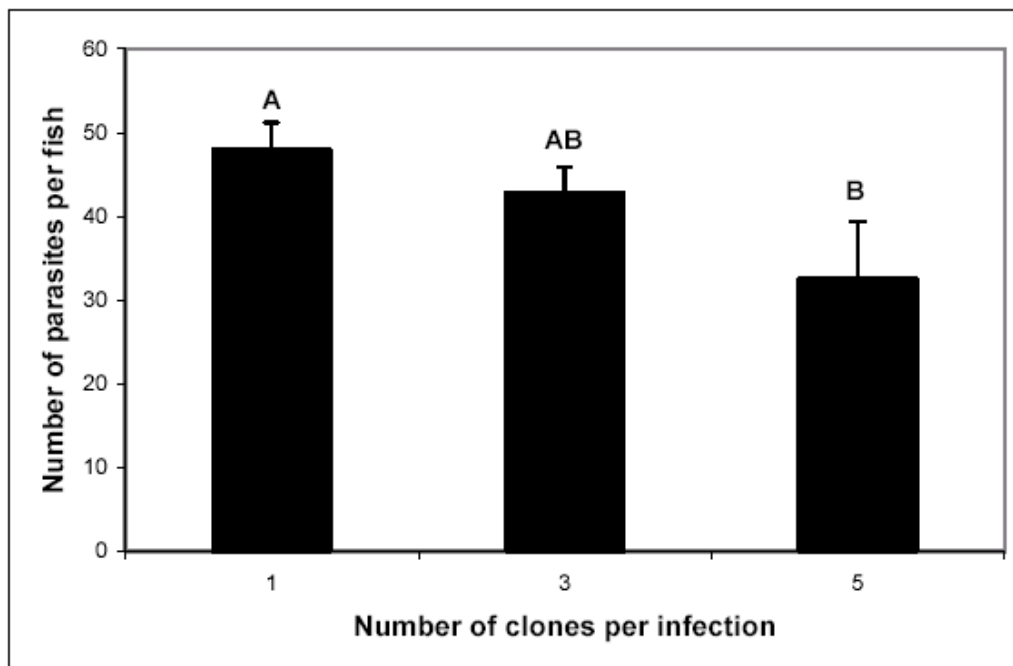


Figure 1-III: Effect of clonal diversity on parasite load. Bars show mean numbers of parasites per fish for each diversity treatment (\pm s.e.). Bars marked with different letters are significantly different.

Selection and complementarity effect

Figure 2-III shows the mean relative numbers of parasites for each clone in the three diversity treatments. Relative number is simply the number found for each clone in each fish divided by the initial number used to infect the fish (150 in the single-clone infection, 50 in the three clone infection and 30 in the five-clone infection). For all five clones, the relative number is greater in the single-clone infection than in the five-clone infection and in the three-clone infection than in the five-clone infection. A stepwise decrease from one to three to the five-clone infections is only interrupted in two cases, where the three-clone infection is slightly higher than the one-clone infection.

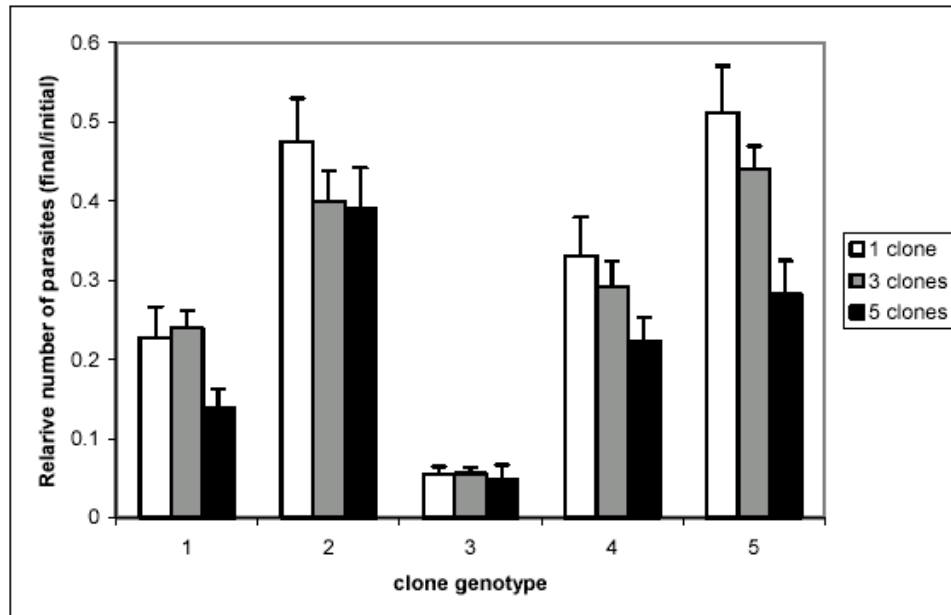


Figure 2-III: Relative number of parasites per clone. Bars show mean relative number of parasites (final/initial) for each clone in the three diversity treatments (\pm s.e.).

The net biodiversity effect was significantly smaller than zero (t-test that intercept of the two-way Anova with factors diversity and fish family is different from zero: $t=-4.58$; $df=130$; $p<0.0001$; figure 3-III). The selection effect was

not significantly different from zero ($t=-1.78$; $df=130$; $p=0.0775$; figure 3-III). In contrast, the complementarity effect was significantly smaller than zero ($t=-2.18$; $df=130$; $p=0.0313$; figure 3-III). For the net biodiversity effect, there was no significant influence of diversity treatment (three-clone or five-clone infections) ($F_{1,130}=2.05$; $p=0.1549$) nor of fish family ($F_{4,130}=0.77$; $p=0.5490$). For the selection effect, there was no significant influence of diversity treatment ($F_{1,130}=0.18$; $p=0.6712$), but fish family had a significant influence ($F_{4,130}=3.10$; $p=0.0179$). For the complementarity effect, there was no significant influence of diversity treatment ($F_{1,130}=2.91$; $p=0.0904$) nor of fish family ($F_{4,130}=0.38$; $p=0.8225$). None of the diversity-fish family interactions were significant.

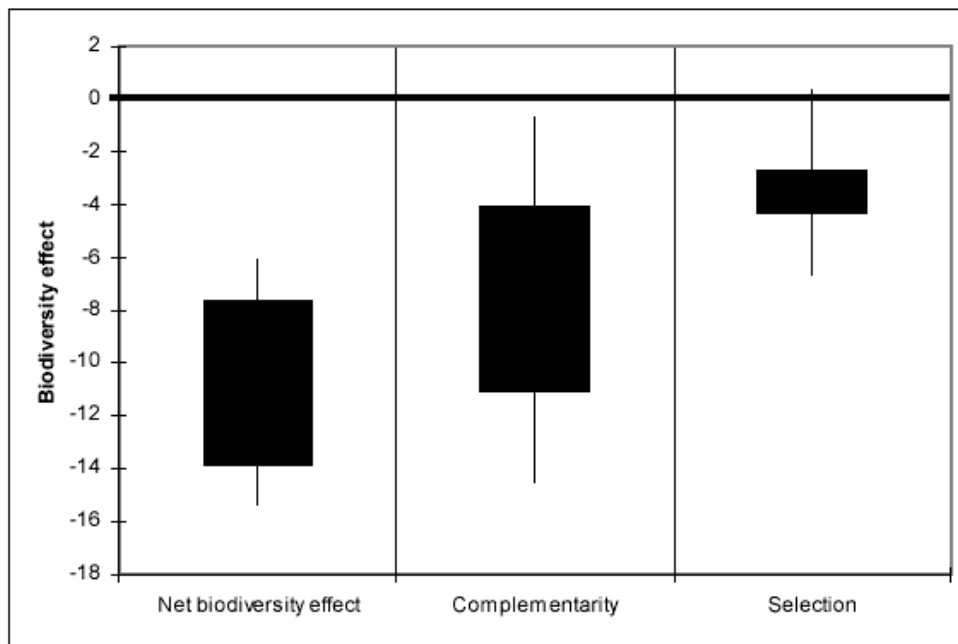


Figure 3-III: Biodiversity effects on parasite number. Black boxes show mean net biodiversity effect, complementarity effect and selection effect, with upper and lower box boundaries representing the standard error. Vertical lines show the 95% confidence interval. Boxes with vertical lines crossing the zero-line are not different from zero.

Restricted defence

We tested whether a host is only able to kill a certain number of individuals of each clone, regardless of the numbers used of each clone for infection. The reason for this test is, that in theory such a restricted defence could also explain a reduced number in the mixed infection compared to single-clone infections without competitive suppression. A host could be able to kill exactly 20 individuals of one clone. In the single-clone infection, this would result in 130 individuals of that clone and in 10 individuals in the five-clone infection. Relative to the initial dose, which is five times higher in the single-clone infection, the single-clone infection would still have more parasites ($130/5=26$) than the mixed-clone infection (10). However, number of parasites killed of each clone clearly depended on the initial infection numbers. In average, hosts were able to kill 23.5 (± 0.57 s.e.) parasites of each clone in the mixed infections, whereas the number of killed parasites was with 102.1 (± 3.83 s.e.) more than four times higher in the single-clone infection (Welch Anova: $F_{1,111}=412.47$; $p<0.0001$).

Discussion

The evolution of virulence is a fascinating and controversially discussed topic. Virulence is the amount of damage a parasite causes to its host (Frank 1996). Several models predict that in mixed-genotype infections virulence evolves to higher levels (Frank 1996; Ebert 1998; Nowak & May 1994; Brown *et al.* 2002). The reason for this increase in virulence is that parasites, which exploit the host more rapidly to increase their reproduction rate, are favoured. A higher exploitation rate may then lead to a greater damage to the host and hence to a higher virulence. However, an enhanced exploitation of the host is not the only way a parasite genotype can outcompete co-infecting genotypes. Suppressing the growth of co-infecting genotypes is another way (Chao *et al.* 2000). If this suppression ability entails a cost for the own growth rate, virulence actually evolves to lower levels in mixed-genotype infections. In our

study, we found exactly such a competitive suppression of co-infecting genotypes proposed as a driving force for the evolution of lower virulence in mixed-infections.

In the natural habitat, mixed-infections are common and nearly all *D. pseudospathaceum* parasites infecting one three-spined stickleback host belong to a different genotype (Rauch *et al.* 2005, chapter 2). So there is ample opportunity for competitive suppression. The interesting question now arises what happens when the genotypic diversity declines. Such a decline is predicted through human caused habitat fragmentation (Young *et al.* 1996). As competitive suppression between different genotypes is decreased, the parasite load of the genetically uniform infection would be higher as the load from a mixed infection, given an identical initial number of infective stages present. The feeding efficiency of whitefish decreased with increasing numbers of a trematode parasite in the eye lenses (Crowden & Broom 1980). Therefore, an increase in parasite numbers due to a decreased parasite diversity may lead to serious fitness decline in fish.

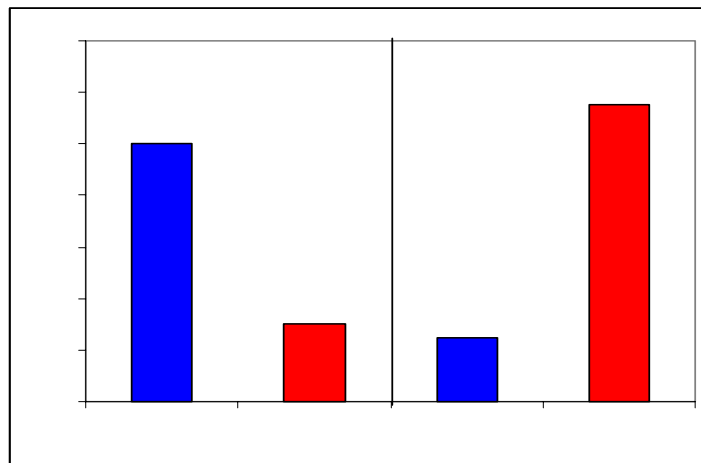
Three possible mechanisms can cause the observed competitive suppression. First, direct suppression of competitors can occur with aid of chemical substances that harm only co-infecting genotypes but not the producing genotype itself, as shown in vitro for viral and bacterial pathogens (Hart & Cloyd 1990; Riley & Gordon 1999). Second, parasites may use the host immune system to suppress competitors. The stickleback immune system is capable of a fast and genotype specific defence (chapter 4). A parasite genotype could produce and release antigens similar to those of a co-infecting genotype into the host environment. The activation of the specific immune defence against the co-infecting genotype would then be stronger compared to the activation caused by the co-infecting genotype alone. Third, parasites could be competitively suppressed by the host immune system in an indirect way. Despite of the genotype specific defence, some degree of cross-reactivity could be possible. Cross-reactivity is the ability of the same immune defence molecule to react against several genetically different parasites (Kurtz

2005). A specific defence directed against one genotype may also have an, although much smaller effect against a different genotype. It was shown that infection with one strain caused protection against a different strain (Berchieri & Barrow 1990; Lipsitch *et al.* 2000). Thus, in the mixed infection, clones may not only suffer under the specific immune defence directed against themselves, but to a lesser extent also from the immune defence directed against co-infecting genotypes.

In summary, we showed here a negative effect of genotypic diversity for parasite fitness and explicitly that this negative effect was due to competitive suppression between different genotypes in mixed infections. As drug treatment success and infection development critically depend on the mechanism at work, we propose here the importance of distinguishing between the complementarity and selection effect for host-parasite interactions.

Chapter 4

The fast and specific defence



Abstract

In vertebrates, the immune defence against parasites is either fast and rather unspecific (innate immune system) or needs several days to become fully mounted and is highly specific (adaptive immune system). Evidence for a fast and at the same time highly specific immune defence is so far lacking. Specificity is defined here as differential defence strength against different parasite genotypes of the same species. Parasites without conserved molecular patterns for being recognized by the innate immune defence, can escape the first defence line. The specific defence could recognise those parasites, but faces a problem: Those parasites may seriously harm the host or escape recognition before the specific defence is ready. A fast and specific immune defence could solve this problem. To study specificity during an early stage of infection, we use a trematode parasite that reaches the immunologically protected eye lens of its stickleback host within 24h. Even with such a short exposure, we found genotype specific host-parasite interactions. Such interactions require a genotype specific defence of the host. Thus, we suggest for the first time that a defence line exists working both in a fast and specific way, long before the MHC-based adaptive immune system is ready.

Introduction

Vertebrates rely on two major lines of immune defence against parasites, the innate and the adaptive immune system (Roitt *et al.* 1989). As a fast first defence line, the innate immune system depends on the recognition of conserved molecular patterns shared by large pathogen classes and is thus not regarded as highly specific (Janeway & Medzhitov 2002). Parasites that lose or lack the conserved molecular patterns can therefore escape innate recognition. The adaptive immune defence, in contrast, is highly specific. A specific immune defence is defined here as differential defence strength against different parasite genotypes of the same species (Frank 2002). The defence strength has to vary between different host genotypes to rule out a simple superiority of one parasite genotype over the other. The adaptive immune system is based on recognition of small protein fractions (peptides) and recognition may vary between different parasite clones and depend on the host genotype (Frank 2002). As only few receptors can recognise each particular antigen, a clonal expansion is necessary to build up an effective adaptive immune defence (Roitt *et al.* 1989). This induction process comes at the cost of a time delay of at least three to five days (Janeway & Medzhitov 2002). To the best of our knowledge, evidence for a fast and at the same time highly specific immune defence in vertebrates is so far lacking. Although invertebrates have long been thought to be devoid of an adaptive immune system, they possess skills typical for it. The immune response can be induced (Moret & Siva-Jothy 2003) and specific memory exists (Kurtz & Franz 2003). How much time this reactions need to be mounted is so far unclear. Transplant rejection studies however suggest that a specific induction also needs some days to develop in invertebrates (Hildemann *et al.* 1977).

A time delay of several days needed for the induction of the specific immune system bears a danger: Parasites, that can escape the innate immune system, may grow substantially or reach immunologically protected body compartments before the specific immune system would finally be ready.

A short exposure period of parasites to the host immune system by means of evasion strategies is common for many parasite species (Chappell *et al.* 1994). To test the influence of the early exposure time, we used as our model system the interaction between three-spined stickleback fish (*Gasterosteus aculeatus*) and the trematode *Diplostomum pseudospathaceum*. The parasite penetrates the skin of the fish and migrates within 24h through the body into the eye lens, where it is protected from the immune system (Chappell *et al.* 1994).

Here we examined whether the apparently contradictory combination of a fast *and* at the same time highly specific immune defence is possible. We did this by testing for genotype specific host-parasite interactions, as they require a genotype specific defence of the host, and a genotype specific infectivity of the parasite (Carius *et al.* 2001). Such specific interactions have been repeatedly found, although only at least six days after infection and do therefore not exclude a time-consuming induction of the defence (Karban 1989; Burdon & Jarosz 1991; Kaltz *et al.* 1999; Schmid-Hempel *et al.* 1999; Carius *et al.* 2001; Mallon *et al.* 2003). As the parasites used in this study are only exposed for 24h to the host immune system, we propose that the defence specificity is too fast to rely on the induction of the MHC-based adaptive immune system or to other time-consuming inducible defences.

Methods

Study system

In the complex life cycle of the trematode parasite *Diplostomum pseudospathaceum*, sexual reproduction takes place in the gut of fish eating birds. Parasite eggs are shed into the water and hatching larvae infect water snails, where asexual reproduction occurs. Snails release free-swimming larvae, which infect fish, such as three-spined stickleback (*Gasterosteus aculeatus*). Infected fish suffer from increased predation risk (Crowden & Broom 1980).

To study clonal variation in infection, we established five clonal lines of the parasite *D. pseudospathaceum*. We isolated parasite eggs from faeces collected from herring gulls (*Larus argentatus*) living in a large colony on an island of an interconnected lake system in northern Germany. We added single hatched larvae to single water snails (*Lymnaea stagnalis*) from a large laboratory breed originating from the same lake system. Successful parasites reproduced asexually within the snails and after two months, snails started to release stages ready to infect fish. All parasites released from one snail belonged to one clone. We included only clones originating from faeces of different gulls.

We only used clones identified morphologically as *D. pseudospathaceum* (Niewiadomska & Kiseliene 1994). Further, we used ribosomal DNA-ITS sequences (Internal Transcribed Spacer) to verify that the clones of *D. pseudospathaceum* used belong to the same species. Non-coding ITS sequences are widely used for phylogenetic analysis due to the within-species homology and between species divergence (White *et al.* 1990). To establish fish families, we caught wild fish from the same lake system, from which the parasite and the snails originated. We produced 10 unrelated F1 fish families. From each F1 family, we used only one fish. We formed five pairs and produced five F2 families (for details on crossing procedure and rearing conditions see Rauch *et al.* 2006, chapter 5).

Parasite infection design

In single-clone infections, we infected fish of each of five families with each of five parasite clones, resulting in a complete cross-infection experiment with 25 fish family-parasite clone combinations. We used five fish of each family for each clone (except for one fish family, where only four fish were used due to small offspring number in that family). We placed the fish singly in 1l aquaria. To each fish, we added 150 parasites of the respective clone into the water not later than 12h after the parasites emerged from the snails. We randomly distributed the aquaria on tables in a climatic chamber (18°C, 16h

light). We allowed the parasites to grow for one week in order to minimize counting mistakes, as the bigger stages are better detectable. We then killed the fish with an excess of methane sulphonate (MS 222), dissected and isolated the eye lens and counted the parasites within the undamaged eye lens.

Data analysis

We analysed the data with a fully crossed two-way ANOVA with clone and family entering as random effects. Numbers were log transformed to restore normal distribution (Kolmogorov-Smirnov test) and equality of variances (Cochran test).

Long-term survival

Although parasites are supposed to be immunologically protected within the eye lens (Chappell *et al.* 1994), we wanted to make sure that no unknown host defence mechanism that may be working within the eye lens, nor parasite competition or nutrient shortage could decrease parasite numbers over time once inside the eye lens. To test for such a decrease, we infected four fish of each of the five families, where each fish was exposed to 150 parasites (30 parasites of each clone). We estimated numbers of parasites for the same individuals one week and nine weeks after infection. An estimation of parasite numbers in living fish is possible by visual examination through the pupil into the eye lens.

Results

We identified a significant fish family-parasite clone interaction ($F_{16,82}=1.97$; $P=0.0249$; figure 1-IV). No parasite clone performed best in all fish families and no fish family had the highest resistance against all clones. Clone identity had an effect on the number of parasites per fish ($F_{4,16}=24.57$;

$P < 0.0001$). Also, fish family influenced number of parasites ($F_{4,16} = 3.88$; $P = 0.0218$).

We did not find a significant decrease in number of parasites from the first to the ninth week after infection (paired t-Test: $t_9 = 0.55$; $P = 0.5960$). This indicates that our findings are not influenced by later acting defence mechanisms. Ten fish died one day after the first check, which was probably due to handling stress during the parasite screen.

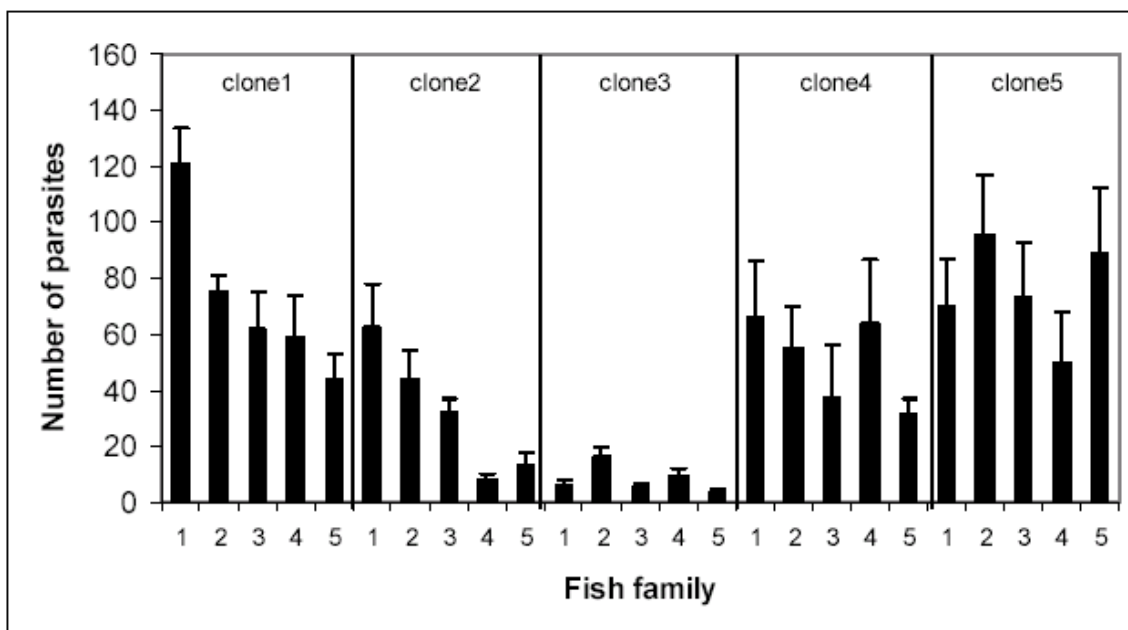


Figure 1-IV: Instant specific interactions between fish families and parasite clones. Bars show mean number of parasites (\pm s.e.) found per fish in the 25 fish family-parasite clone combinations.

ITS analysis showed that a 566 base-pair fragment of the first spacer and a 214 base-pair fragment of the second spacer, which excluded the anyway highly conserved 5.8S ribosomal gene, were 100% identical for all five clones (three replicates per clone). An NCBI BLASTn search using the

more frequently analysed first spacer fragment showed that there were zero nucleotide differences compared to a sequence from *D. pseudospathaceum*, but four changes compared to the next closest match (*Diplostomum indistinctum*).

Discussion

Genotype specific host-parasite interactions are the basis for the Red Queen hypothesis and co-evolution theory (Hamilton 1980). This study showed for the first time genotype specific host-parasite interactions that are initiated within a day or less. Such interactions require a specific defence. The suggested fast and specific immune defence is novel, as the immune defence was thought to be either fast and unspecific or slow and specific. A fast and specific defence has great advantages. Parasites, otherwise not recognised by the relatively unspecific innate immune system relying on conserved molecular patterns, can quickly be eliminated before they damage seriously the host by a high multiplication or growth rate or reach immunologically protected host body parts. This adds to the recent debate on the complexity of immune mechanisms not relying on the induction of the MHC-based adaptive immune system alone, but on other as yet unknown molecular mechanisms (Little *et al.* 2005; Kurtz 2005).

Possible candidates for the underlying molecular mechanism include lectin-like receptors, which recognise surface sugar compounds on the parasite surface (Roitt *et al.* 1989). Surface sugar composition on different parasite clones may differ. This could lead to a different binding affinity by the lectin-like receptors to different clones, which may change depending on the host genotype. The difference in binding affinity may then determine the strength of the defence (Roitt *et al.* 1989). Such a specific defence would be instantly ready. Prompt, yet specific, reactions have been demonstrated for

sperm-egg interactions, which are mediated by surface molecules such as the protein bindin (Evans & Marshall 2005).

Although the underlying molecular mechanism remains to be investigated, experimental evidence at the phenomenological level is an essential first step, before uncovering the exact molecular pattern (Kurtz 2005). This guideline was successful in exploring, for example, the adaptive immune system and pathogen specific responses in vertebrates (Little *et al.* 2005). In this study, we suggest for the first time that a defence line exists working both in a fast and specific way, long before the adaptive immune system is ready.

Chapter 5

The role of the adaptive immune system



Abstract

We tested how major histocompatibility complex (MHC) genes influence parasite load in a natural experiment. We crossed three-spined sticklebacks from a river habitat with genetically distinct lake sticklebacks and intercrossed the resulting F1 hybrids. F2 offspring segregate into pure river or pure lake MHC genotypes and into two hybrid genotypes, while randomising the background genome across all MHC genotypes. In outdoor cages, we exposed fish reciprocally to the natural parasite communities of the river and lake habitats. MHC genotype did not significantly influence parasite load. In contrast, genomic background explained a significant percentage of the variation in parasite load.

Introduction

Genes of the major histocompatibility complex (MHC) play a key role in the activation of the adaptive immune system in vertebrates (Janeway *et al.* 1999). The great interest devoted to the impact of MHC genes on parasite resistance is therefore not surprising. Specific MHC alleles are associated with disease resistance or susceptibility (reviewed in e.g. Apanius *et al.* 1997). In three-spined sticklebacks, individuals with intermediate numbers of MHC alleles had the lowest parasite load (Wegner *et al.* 2003a and b) and parasite growth (Kurtz *et al.* 2004). MHC congenic mice, i.e. inbred strains that differ only in their MHC haplotype, have been widely used to experimentally test the influence of MHC genes on resistance to various diseases (reviewed in Apanius *et al.*, 1997). However, differential accumulation of mutations in different strains can constrain conclusions made from experiments with congenic animals (Carroll and Potts 2001). Experiments using second-generation mice (F2) obtained from crosses between different parental strains and intercrossing the F1 generation to extract the original MHC haplotypes, avoid this problem by randomising any background mutations. Such studies demonstrated the effect of MHC genes on pathogen susceptibility (Penn *et al.* 2002; Lohm *et al.* 2002).

Despite the interest in MHC genes, no direct experimental evidence exists showing the influence of MHC genes on parasite load in a natural habitat. An experiment in the natural habitat has the obvious advantage of covering the full range of natural parasite species. Using only single parasite strains or species can be problematic, as the fitness of an individual parasite might depend on potentially co-infecting parasites (Brown *et al.* 2002). Furthermore, whether or not a difference in susceptibility between different MHC haplotypes exists or not depends on the parasite species (reviewed in Apanius *et al.* 1997). In a laboratory experiment, the number of MHC alleles only influenced parasite load when exposing fish to several parasite species (Wegner *et al.* 2003a). Furthermore, an experiment in the natural habitat

avoids possible artificial conditions of laboratory experiments. At the same time, it preserves the strength of an experimental manipulation lacking in correlative studies.

Three-spined sticklebacks (*Gasterosteus aculeatus*) present an ideal system to test whether MHC genes influence the parasite load in the field. We take advantage of populations derived from river and lake habitats, since river and lake sticklebacks have almost completely divergent MHC genotypes, as revealed by sequence comparison (Reusch T.B.H., Wegner K.M., Kalbe M. and Stam, W.T., unpublished data). Gene flow between the two habitat types is practically absent (Reusch *et al.* 2001b). This genetic divergence allowed us to obtain replicated F2 siblings with completely divergent MHC genotypes by crossing river fish with lake fish and intercrossing the F1 hybrids. In each family, F2 offspring will segregate into pure river and pure lake MHC genotypes as well as into the two possible hybrid MHC genotypes that carry a common background genome randomised over MHC genotypes. In exposing the F2 fish to the natural parasite communities in the river and lake, we tested for an effect of MHC genotype on parasite load in the natural habitat. In using several hybrid families, we tested the effect of different background genomes (=family effect). The parasite communities in the two habitat types differ, with lake fish harboring more parasite species than river fish (Kalbe *et al.* 2002).

Genes closely linked to the MHC region do not segregate independently from the MHC genes during meiosis. For three-spined sticklebacks, Peichel *et al.* (2001) showed that 26 linkage groups exist. For example, MHC class I and II genes are on different linkage groups (Sato *et al.*, 2000), allowing the effects of the two classes to be separated. In this study, we concentrated on the MHC class II genes, as they are critical for activation of the immune system against most extracellular parasites (Penn & Potts 1999). Furthermore, crossing-over events (recombination) within the MHC class II do happen (Reusch *et al.* 2004, chapter 1). Thus, the combined effect of segregation and crossing-over should result in an acceptable randomisation level of the background genome over the MHC genotype.

Aside from testing whether the difference in MHC sequence influences parasite load, we also tested whether MHC genotypes are locally adapted, as organisms are often found to be locally adapted (Lively 1989; Ebert 1994). Additionally, we tested whether there is a “general” local adaptation of the fish to their natural habitat not restricted to the MHC genotype. To answer this question, we also exposed pure lake and river lines, bred over two generations, reciprocally in the two habitats.

Despite the important role of MHC genes for parasite resistance, the existing evidence for MHC dependent parasite resistance in natural populations is only correlative. In decoupling the MHC genotype from the background genome, our experimental design allowed us to explicitly study the influence of MHC genotype on parasite load and of MHC origin for local adaptation in a natural habitat.

Methods

Fish rearing conditions

We caught three-spined sticklebacks from one river (the Schwale river) and one lake (the Vierersee lake) from two different drainage systems in northern Germany in autumn 2002. We kept fish in 16 l tanks at a density of around 20 fish/tank with continuous water exchange in “winter” conditions (6°C, 8h light). We fed the fish with frozen red mosquito larvae. In spring 2003, we transferred fish for two weeks to “spring” conditions (12°C, 12h) and subsequently to “summer” conditions (18°C, 16h). We isolated males in 16 l tanks and offered artificial nesting material. We kept females at a density of five fish/tank. We introduced a female ready to spawn with the male and after successful mating, we transferred eggs to individual tanks. We fed the hatching offspring of the F1 generation with live *Artemia salina* for the first two months, and we kept all offspring of one family in one tank. After this period, we fed fish with frozen red mosquito larvae, and kept fish at a density of around 20 fish/tank. In autumn 2003, we passed F1 fish through an artificial

winter (two weeks in “spring” conditions, followed by four weeks in “winter” conditions, then again two weeks in “spring” conditions) and subsequently transferred the fish to “summer” conditions for breeding of F2 offspring. After seven months of growth in “summer” conditions at a density of around 20 fish/tank, we used these F2 fish for the experiment.

Breeding design

Figure 1-V shows the breeding design we used to obtain fish with different MHC genotypes and a randomised background genome over MHC genotypes. We produced eight F1 hybrid families. Each hybrid family originated from a cross between a wild-caught river fish and a lake fish. Each parental fish was only used once. In four crosses, the males came from the river and the females from the lake and in the other four crosses, the males came from the lake and the females from the river. From each of the resulting eight hybrid F1 families, we chose one fish, either a male or a female. We used each of the eight fish only once to form four male-female pairs to produce four F2 families (crosses took place only between fish where both had either a river father or both had a lake father). F2 fish from these four families are hereafter denoted as hybrid families. Fish with both MHC haplotypes originating from river fish are referred to as RR fish, fish with two lake haplotypes as LL fish and fish with mixed haplotypes as LR. How we identified the different MHC genotypes is described below. In contrast to these three classes of MHC origin (LL, RR and LR), 16 possible genotypes exist in the four F2 hybrid families, four in each family. The genotypes are characterised as LL_n , RR_n , LR_n and RL_n with n as family number 1-4. 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 stand for the lake or river origin of the haplotype. As the MHC genotypes differ almost completely in the two habitats, this design resulted in F2 offspring segregating within each family into four distinct MHC genotypes with a shared randomised background genome.

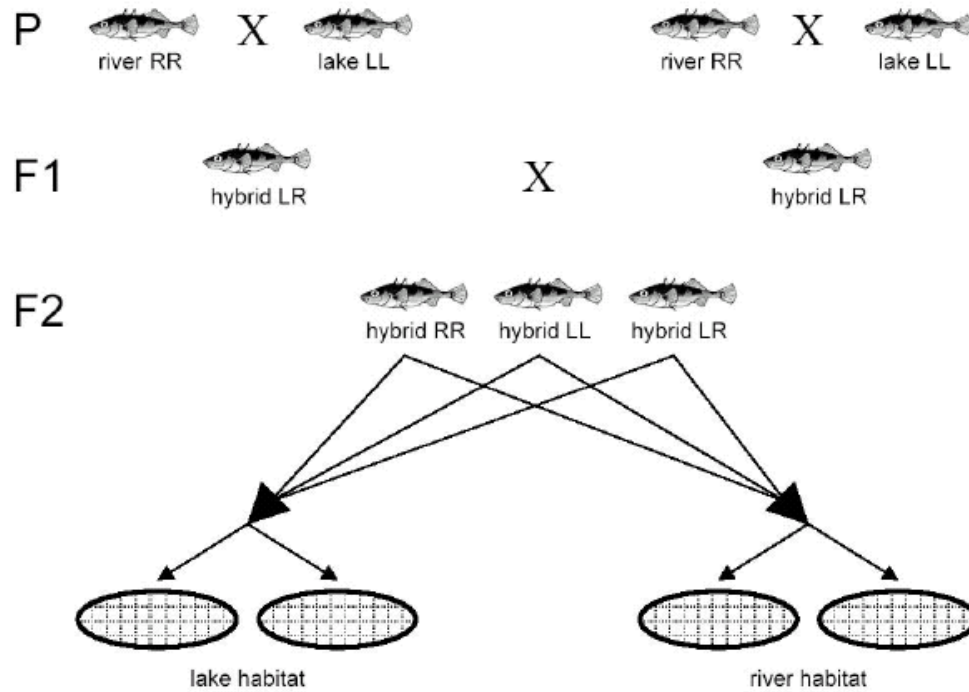


Figure 1-V: Breeding design of one hybrid family. River, lake or hybrid fish describe the origin of the background genome. The capital letters stand for the origin of the MHC: RR for river MHC, LL for lake MHC and LR for hybrid MHC from river and lake. Arrows indicated the assignment to the four cages (ovals).

To further obtain pure river fish, we produced six crosses between wild-caught river fish. From each of the six F1 families, we chose one fish. We used each of the six fish only once to form three pairs to produce three F2 pure river families (referred to as PR fish). The same procedure with lake fish yielded three F2 pure lake families (referred to as PL fish).

The field exposure

Within a single hybrid family, we selected five to eight fish (numbers depended on the family) with an LL MHC origin, five to eight fish with an RR MHC and five to eight fish with an LR MHC (MHC genotyping explained

below). We intermixed the selected fish of all three MHC origins in a single aquarium. Then we randomly distributed the fish into one of two cages and placed the cages in the lake (figure 1-V). From the same family, we intermixed five to eight fish of each MHC origin in a second aquarium, and randomly distributed to two cages and placed the cages in the river (figure 1-V). We repeated this procedure for the remaining three hybrid families by distributing each family to four new cages (16 cages in total). This resulted in having only fish of the same hybrid family in the same cage, so that the three different MHC origins and the four genotypes of the same family experienced the same environment. Any cage effect was thus randomised over the four cages used for each family. Numbers of fish with the same MHC origin included per family and habitat varied from five to eight as different MHC origins were not equally available. Fish with an LR MHC origin included two different genotypes within each family: LR n and RL n MHC genotype. The two genotypes were not represented in equal numbers within each family. Per family we included six to 13 LR n fish and two to six RL n fish. On average, a single genotype was represented by 9.1 fish distributed in the two habitats.

Additionally, we released in every cage, two fish of each pure river family and of each pure lake family. This resulted in having 14 to 16 fish in each cage. Prior to release, we measured size and weight for every fish.

We released the F2 fish in cages placed in the river and lake, near the two catching areas of the parental generation, in August 2004. In the river, we placed eight cages at intervals of 10 metres in the middle of the river (the river bed is two metres wide and half a metre deep). In the lake, we placed eight cages 10 metres offshore at 1 metre depth in intervals of 10 metres. Cages were pipe shaped (1m length and 40cm diameter) and covered with wire netting with 4mm mesh size.

MHC genotyping and individual characterisation

We extracted DNA of each F2 individual from a small piece of dorsal spine as described in Reusch *et al.* (2001b). To identify the different MHC

class II genotypes we combined the discrimination effect of the binding specificity of eight newly developed primers by using the single-strand conformation polymorphism (SSCP) method. Each of the eight primers used amplifies a unique subset of MHC class IIB sequences. We further separated each subset using single-stranded conformation polymorphism (SSCP) on a Capillary Sequencer (Binz *et al.*, 2001). For details of the primer development and PCR conditions see Wegner (2004). We preferred the eight new primers over the two old primers used in Reusch *et al.* (2001a), Wegner *et al.* (2003a and b) and Kurtz *et al.* (2004), because with the two old primers, we could not distinguish all four genotypes in all four hybrid families. For simplicity, we refer to different sequences as being different alleles although they may stem from different loci. With aid of this newly developed technique, within each hybrid family we can distinguish all four possible MHC genotypes.

Using five polymorphic microsatellites, every experimental fish got an individual identity to discriminate them after the experiment for measuring growth and change in weight during the experiment. We used the microsatellites Gac1097, Gac1125, Gac4170, Gac5196 and Gac7033 developed by Largiader *et al.* (1999) and modified by Reusch *et al.* (2001b).

Parasite load

We collected fish eight weeks after release. At that time point, fish were still sexually immature. We measured size and weight again, and we checked fish for parasite infection. We screened the surface and all inner organs, including eyes and gills (for details on parasite screening see Kalbe *et al.* 2002). We found fifteen macroparasites and recorded their numbers quantitatively except for *Trichodina sp.* (table 1-V). For *Trichodina sp.* (Ciliata), we estimated the number of parasites to be either 0, 10, 50 or 100 on the visible parts of the fins. An estimation of *Trichodina sp.* numbers was possible, as fish were not heavily infected and numbers did not exceed 100 individuals per fish. Both *Trichodina sp.* and *Gyrodactylus sp.* (Monogenea) can

reproduce on the fish. Therefore, numbers found on the fish are probably not equivalent to numbers actually infecting the fish.

Parasite species	class	River habitat			Lake habitat		
		P%	mean	max	P%	mean	max
<i>Proteocephalus filicollis</i>	Cestoda	27.27	0.52	8	5.88	0.06	1
<i>Trichodina sp.</i>	Ciliata	2.48	0.25	10	68.91	21.85	100
<i>Anguillicola crassus</i>	Nematoda	0.83	0.01	1	15.97	0.21	3
<i>Raphidascaris acus</i>	Nematoda	13.22	0.17	3	0.84	0.02	2
<i>Gyrodactylus sp.</i>	Monogenea	69.42	4.6	38			
<i>Acanthocephalus lucii</i>	Acanthocephala				0.84	0.01	1
<i>Paradilepis scolecina</i>	Cestoda				2.52	0.03	1
<i>Valipora campylancristota</i>	Cestoda				20.17	0.39	5
<i>Argulus foliaceus</i>	Crustacea				12.61	0.18	4
<i>Apatemon cobitidis</i>	Digenea				1.68	0.03	2
<i>Cyathocotyle prussica</i>	Digenea				5.04	0.07	3
<i>Diplostomum sp.</i>	Digenea				100	18.8	64
<i>Echinochasmus sp.</i>	Digenea				18.49	0.19	2
<i>Tylodelphis calvata</i>	Digenea				6.72	0.24	11
<i>Camallanus lacustris</i>	Nematoda				23.53	0.32	4

Table 1-V: Table shows parasite species name, class name, prevalence in % (P%), mean abundance per fish (expressed as average number of individual parasites per fish, including zero counts for fish not infected with the respective parasite species), and maximal numbers found on a single fish (max) for the two habitats separately.

For every individual fish we calculated an overall parasite load averaged over all parasite species. Some parasite species occur at much higher numbers per fish than other species. Such species would largely influence the overall parasite load. To correct for this effect, we used relative numbers for a given parasite species and habitat, calculated as follows. For each parasite species, we recorded the maximal number found on a single fish in the experimental population separately for both habitats. Then we divided number of individuals of one parasite species found on one fish with the maximal number of the corresponding parasite species. We did this for every parasite species and habitat type separately. Fish not infected by one

parasite scored a zero for that parasite species. To calculate the parasite load of one fish we took the average over all relative numbers of parasite species actually present in the respective habitat. We also calculated the parasite load using the Shannon diversity index calculated separately for each fish (Magurran 1988) and a normalized value (for every fish, the deviation from the mean abundance of a given parasite species in a given population was expressed in SD units and averaged over all parasite species present in a given habitat). The results obtained with the two alternative parasite loads were consistent with the original parasite load calculated with relative numbers and are not reported.

To test whether the MHC genotype had an influence on particular parasites in the hybrid lines, we focused on two parasite species with a high prevalence (>60%), the specialist *Gyrodactylus sp.* (river habitat) that is mainly found on three-spined sticklebacks and the generalist *Diplostomum sp.* (lake habitat) that also infects several other fish species (table 1-V). We then analysed two parasite species with a low prevalence (<30%), the specialist *Proteocephalus filicollis* (analysed solely for the river habitat, as only five fish were parasitised by this species in the lake habitat) and the generalist *Valipora campylancristrota* (lake habitat).

Data analysis

We tested the influence of the 16 different MHC genotypes in the hybrid lines on parasite load by nesting the MHC genotypes within the family effect. We tested this ANOVA using the residuals taken from a one-way ANOVA with habitat as fixed factor to control for habitat effect. We treated MHC genotype and family as random factors. To test for MHC local adaptation in the hybrid lines, we determined the interaction between origin of MHC (LL or RR) and the habitat of exposure from an ANOVA model. MHC origin was fully crossed with the factors habitat and family. To test for local adaptation in general, we measured the interaction between origin (lake, river or hybrid lines) and the habitat of exposure. Family effect is nested within

origin, but not within habitat of exposure, resulting in a split-plot design. Family effect is controlled for in this design, but family effect statistics are not shown, as drawing conclusions from the nested factor statistics is not recommended in such a split-plot design. We further tested for a correlation between number of MHC alleles and parasite load. We tested the correlations using the residuals obtained from a two-way ANOVA including the factors habitat of exposure and family as described in Wegner *et al.* (2003a). We first did this correlation with pure lake lines only and then for all fish, as Wegner *et al.* (2003a) and Kurtz *et al.* (2004) also used lake lines in their experiments. For all analyses we tested residuals graphically for normal distribution and where necessary we used log or square root transformations.

Results

MHC genotypes

In total, we found 38 different SSCP signals each probably corresponding to different MHC alleles in the experimental fish. We did not find any crossing-over events within the MHC region. In the hybrid lines, we could clearly distinguish the four possible MHC genotypes within each family in all cases. Also considering each of the eight primers singly, MHC genotypes differed markedly within each family. In three families, RR and LL MHC genotypes did not share any alleles and in one family only one allele was shared. Comparing over all four families, MHC genotypes differed from each other at least for two alleles, except for one case of two identical LL MHC genotypes.

In the pure lines, MHC genotypes did not differ within each family as markedly as in the hybrid lines. In three families, we could only distinguish two or three different MHC genotypes of the four possible genotypes in F2 fish. In case of the pure lines, we could not exclude the possibility that our MHC discrimination technique failed to distinguish between different MHC sequences. We therefore used the pure lines solely to test for “general” local adaptation and not to test for an influence of MHC genotype on parasite load.

Fish condition

In the hybrid lines, MHC origin (RR, LL or LR) had no significant influence on fish-length or fish mass before the start of the experiment (length: $F_{2,115}=0.02$, $p=0.9796$; weight: $F_{3,114}=0.40$, $p=0.6737$). Of the 242 released fish, 11 died in the river cages, and 12 in the lake cages, during the experiment. Neither changes in fish-length ($F_{1,214}=0.20$, $p=0.6561$) nor in fish weight was correlated with parasite load ($F_{1,214}=0.06$, $p=0.7993$). Details of the parasite infections are summarized in table 1-V.

Influence of MHC genotype on total parasite load

The different MHC genotypes within each family did not significantly influence parasite load (figure 2-V). The null hypothesis of no influence of the MHC genotype on parasite load was not rejected with a power of 56% (at $\alpha = 0.05$). Only one MHC genotype, RL4 in the fourth family represented by only two fish, seemed to have a higher parasite load than the other genotypes. In contrast, family specific genetic background did influence parasite load (figure 2-V). Family factor explained 8.36% of the total variance and MHC genotype 0.16%. The family raw-effect size ($=0.027$) is 1.8 times higher than the raw-effect size of MHC genotype ($=0.015$). Details of this ANOVA with MHC genotype nested within family and controlling for habitat effects are shown in table 2a-V.

Influence of MHC genotype on parasite load of single species

MHC genotype had no significant effect on the number of all four parasite species analysed separately (table 2b-e-V). Consistent with the results obtained with the total parasite load, family had in three of the four cases a significant effect (table 2b-e-V).

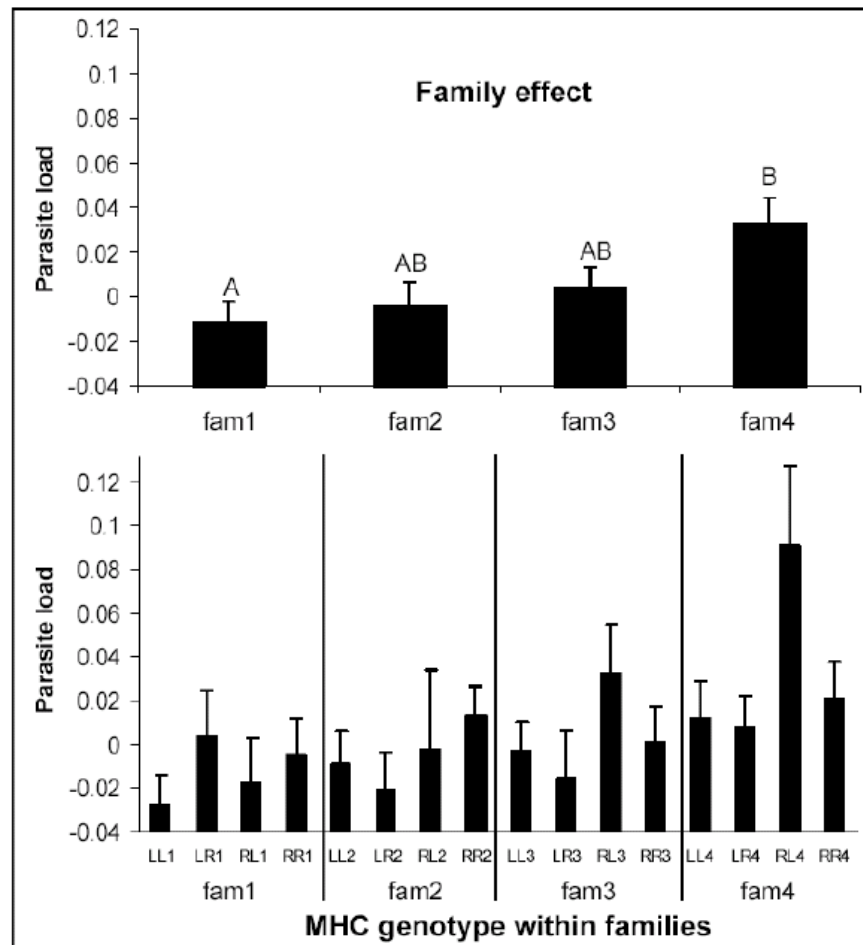


Figure 2-V: Effect of family and MHC genotype on parasite load. The upper panel shows mean parasite load of sticklebacks in the four hybrid families, exposed in the two habitats. Bars marked with different letters are significantly different after Tukey HSD multiple comparison test. The lower panel shows the parasite load for the 16 MHC genotypes in the four hybrid families. Bars show least square means (\pm s.e.), computed from residuals of an ANOVA including habitat effect.

Local adaptation

We did not find any evidence for MHC local adaptation in the hybrid lines, i.e. no statistically significant interaction existed between habitat of

exposure (river or lake) and origin of MHC (RR or LL) (figure 3-V). Parasite load was higher in the lake habitat than in the river habitat. This was not a surprise, as the mean abundance of the different parasite species was much higher in the lake habitat than in the river habitat. MHC origin did not influence parasite load nor was there a family effect. Details of this fully crossed 3-way ANOVA are shown in table 2f-V. The parasite load of fish with a mixed MHC from the river and the lake (LR) did not significantly differ from the fish with an LL or RR MHC ($F_{2,117}=1.04$, $p=0.3571$, habitat and family effect included in the model, statistical details not shown).

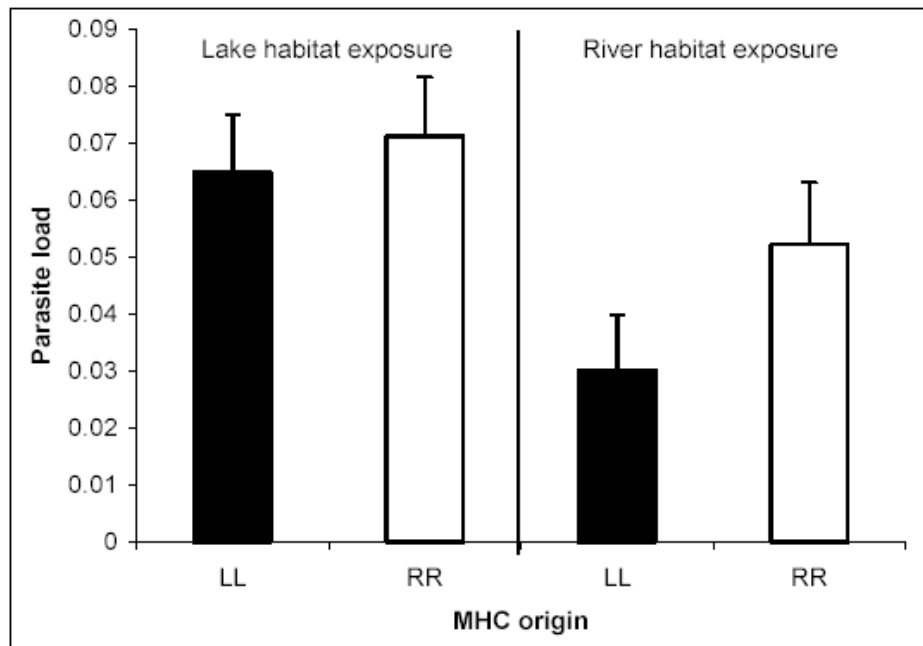


Figure 3-V: Local adaptation of MHC origin. Parasite load of F2 hybrid sticklebacks, carrying MHC genotypes of different origin (LL = lake, RR = river) and exposed to parasites in the two habitat types. Bars show least square means (\pm s.e.), controlling for family effects.

In a test for “general” local adaptation using the pure lines, we also did not find a significant interaction between habitat of origin (pure lake or pure river) and habitat of exposure. Habitat of exposure had no significant effect. Fish from the pure river lines had a significantly higher parasite load than fish from pure lake lines. Statistical details of the split-plot design are shown in table 2g-V. There was also no significant interaction between habitat of origin and habitat of exposure using the pure lines and the hybrid lines (habitat*general origin: $F_{2,226}=2.08$, $p=0.1270$, habitat, general origin and family effect included in the model, statistical details not shown).

Effect of MHC genotype and family on parasite load

	Factor	d.f.	F	p
a) Combined parasite load	MHC genotype	12,125	1.01	0.4397
	family	3,20	3.27	0.0423
b) <i>Gyrodactylus sp.</i>	MHC genotype	12,55	0.53	0.8855
	family	3,29	1.23	0.3158
c) <i>Diplostomum sp.</i>	MHC genotype	12,54	0.98	0.4804
	family	3,21	10.42	0.0002
d) <i>Proteocephalus filicollis</i>	MHC genotype	12,55	0.33	0.9802
	family	3,40	5.62	0.0026
e) <i>Valipora campylancristota</i>	MHC genotype	12,54	1.22	0.2924
	family	3,19	3.60	0.0322

Local adaptation

	Factor	d.f.	F	p
f) MHC	habitat	1,76	6.83	0.0108
	MHC origin	1,76	1.91	0.1705
	family	3,76	1.62	0.1913
	habitat*MHC origin	1,76	0.60	0.4422
	habitat*family	3,76	2.50	0.0655
	MHC origin*family	3,76	0.07	0.9748
	habitat*MHC origin*family	3,76	1.75	0.1641
g) Background	habitat	1,90	2.37	0.1269
	general origin	1,4	7.82	0.0484
	habitat*general origin	1,90	2.63	0.1083

Table 2-V: Statistical details for MHC genotype and family effect and for the local adaptation analysis.

Number of MHC alleles

Fish of the hybrid lines with an RR MHC had in average 6.5 ± 0.20 (\pm s.e.) different alleles: LL MHC fish had 6.0 ± 0.23 alleles; LR MHC fish had 8.4 ± 0.18 alleles; and RL MHC fish had 7.4 ± 0.16 alleles. Pure river fish had on average 6.9 ± 0.24 different alleles, lake fish 7.3 ± 0.27 . Number of different MHC alleles in the pure lake lines only did not correlate with parasite load in either a linear ($F_{1,38}=0.23$; $p=0.6374$) or in a quadratic polynomial way ($F_{2,37}=0.43$; $p=0.6554$), controlling for both habitat and family effects. Also using all fish, numbers did not correlate linearly ($F_{1,224}=0.04$; $p=0.8512$) nor in a quadratic way ($F_{2,223}=0.78$; $p=0.4619$).

Parasites in wild-caught fish

To compare parasite infection of the experimental fish with naturally occurring fish, we caught 30 wild fish near the cages, 15 in the river and 15 in the lake, during the experimental period and screened for parasites. Thirteen of the 15 parasite species found in wild fish we also recorded for the experimental fish. The maximal numbers found in single wild fish exceeded numbers found in single experimental fish for only two parasite species.

Discussion

Despite the increasing interest in the relevance of MHC genes for resistance against parasites, experimental evidence for an effect of MHC genotype on parasite load in natural habitats does not exist. Here we performed an experiment where we exposed sticklebacks to the natural parasite fauna of a river and a lake habitat. Contrary to expectations, we did not find any influence of the MHC genotype on parasite load. Instead, we observed a greater importance of the genomic background for explaining parasite load compared to the MHC genotype.

To test whether MHC genotype has an influence on parasite load of a diverse and naturally occurring parasite community, two basic conditions have to be met. First, experimental individuals have to differ in their MHC genotype independent of their genetic background. We achieved this in a two-generation breeding design, where we obtained replicated F2 fish that belonged to the same family but had completely different MHC genotypes. Second, experimental individuals must be challenged by a diverse natural parasite community. This was achieved by releasing the experimental fish into field enclosures placed in the natural habitats. Parasite loads of experimental fish were very similar to those of naturally occurring fish, as we recorded 13 of the 15 parasite species found in wild fish also for the experimental fish. The parasite community did not only comprise several parasite species, but the within-species diversity was probably also high. For example, we showed that natural occurring three-spined sticklebacks from a lake harboured in average 22 different clones of *Diplostomum pseudospathaceum* per fish (Rauch *et al.* 2005, chapter 2).

The vertebrate immune system can be subdivided into the innate and adaptive immune system (Janeway *et al.* 1999). The innate immune system is a fast and effective first defence line against many pathogens, but is regarded as rather unspecific (Dixon & Stet, 2001). However, recent studies indicate that invertebrates, thought to rely only on innate immunity, possess a specific defence and memory (reviewed in Kurtz 2005). The adaptive immune defence achieves its extraordinary specificity by somatic rearrangement of genes that encode antigen receptors, i.e. T-cell receptors and antibodies (Kurtz 2005). For the activation of an adaptive immune response, genes of the MHC play a key role because they present peptides derived from pathogens to T-cells. The MHC can be subdivided in two main classes. MHC class I encoded proteins mainly present peptides of intracellular parasites, such as many viral or bacterial pathogens (Penn & Potts 1999). This normally results in destruction of the infected cell by cytotoxic T-cells. By contrast, MHC class II encoded proteins mainly present peptides derived from extracellular parasites,

such as extracellular bacteria and macroparasites (Penn & Potts 1999). If the foreign peptide bound to the MHC protein is recognised by T-cells, a complex immune reaction activation is initiated. Obviously, MHC class II genes are an important part of the immune system, yet they are only one part in the network. A whole variety of different mechanisms and pathways exists for defence against pathogens. In this study the background genome had a greater influence in explaining parasite load compared to the MHC class II genes. This influence of the background genome may involve parts of the MHC class II immune reaction cascade that are not necessarily genetically linked to the MHC region. It might further comprise other parts of the adaptive immune system, of course also the innate immune system, as well as interactions between different mechanisms and pathways.

Interactions between MHC genes and genetic background can lead to dramatic changes in parasite resistance (Apanius *et al.* 1997). Such interactions between MHC genes and the genetic background alone is not sufficient to explain the family effect found in this study. For every genetic background, our design had four different MHC genotypes. This leads to a randomisation of the MHC genotype effect over the family effect. Furthermore, it has been shown that products produced by the innate immune system influence the adaptive immune system (Belardelli & Ferrantini 2002). Through the randomisation of the background genome over the MHC genotypes, parts of the river genetic background get connected with lake MHC genotypes and vice versa. If the combination of river and lake parts of the immune system prevents the activation of the MHC genes, no influence of the MHC genotypes on parasite load could be expected. However, in that case we would expect that the pure river and lake lines would have a lower parasite load than the hybrid lines, which was not the case. Studies comparing the relative importance of MHC genes on resistance against single parasite species reported that MHC haplotypes explained between 1.6% and 14% of the total phenotypic variance found (Apanius *et al.* 1997). In our study, MHC genotype

did not explain a significant percentage of the variance in parasite load in contrast to genetic background, which accounted for 8.36% of the variance.

Experiments with congenic mice revealed, that a specific MHC haplotype either increased or decreased susceptibility depending on the parasite population used (Apanius *et al.* 1997). If in this study a certain MHC genotype conferred an increased resistance to some parasite species while simultaneously decreasing resistance to another, the combined parasite load measure would yield an intermediate value. However, for the four parasite species analysed separately, we did also not find an influence of MHC genotype on parasite load.

Numerous papers have shown an influence of MHC genes on parasite load (reviewed in Apanius *et al.* 1997). In this paper, however, we did not find such an influence. In order to rule out a false negative finding, we also have to consider possible limitations of the experimental design. First, the experimental period of eight weeks might have been too short to result in a marked influence of the MHC genotype on parasite load. MHC genes are part of the adaptive immune system, which needs time to build up a defence. However, in a laboratory experiment, which showed an influence of the number of MHC alleles on parasite load in sticklebacks, the time between the first and last infection was also eight weeks (Wegner *et al.* 2003a). Furthermore, in this experiment, the immune system was only challenged for two times with parasites. In our study, parasite challenge was probably continuous, so that the adaptive immune system received many signals for activation. Second, if parasite infections might have occurred almost exclusively in the last weeks of the experiment, the adaptive immune system would not have enough time to get activated. However, parasite infection is probably more likely to decrease when approaching the winter season as water temperature decreases. For example, infection rate of the trematode *Diplostomum spathaceum* in rainbow trout decreased with decreasing water temperature in a laboratory experiment (Stables & Chappell 1986). Third, if the parasite pressure would decline dramatically in the last weeks of the

experiment, there would be very few new infections during the time when the adaptive immune system is activated. This would imply that the parasites we found infected the fish very early in the experiment and that they persisted within the fish until the end of the experiment. This would, however, give the adaptive immune system the opportunity to react against those parasites in the last weeks of the experiment, when it had enough time to build up a defence. Fourth, fish residing inside the cages might have experienced a lower exposure to parasites than free-living fish, potentially resulting in insufficient parasite pressure. This seemed not to be the case, as the parasite load of the experimental fish was very similar to free-living fish caught during the experimental period.

We did not find local adaptation of parasites to local MHC genotypes, as fish with a MHC originating from the river (RR) had in both habitats a slightly higher parasite load than fish with a MHC originating from the lake (LL). Similarly, we did not find a “general” local adaptation using the pure lines, as pure river fish had a higher parasite load than lake fish in both habitats. As one possible explanation, we assume that lake fish are, in general, exposed to a higher parasite pressure, as the number of parasite species is higher in the lake habitat (Kalbe *et al.* 2002). As a consequence, lake fish may just have in general a better defence against parasites without being specially adapted to their home parasites. Kalbe & Kurtz (2006) showed that sticklebacks from a lake population had a higher immunocompetence (respiratory burst activity and spleen size) than sticklebacks from a river population.

Two recent laboratory experiments with sticklebacks have reported that fish with an intermediate number of MHC alleles had the lowest parasite load or parasite growth (Wegner *et al.* 2003a, Kurtz *et al.* 2004). In a field experiment, Wegner K.M. Kalbe M. and Reusch T.B.H. (unpublished data) showed, that fish with an intermediate number of alleles had the highest survival rate. Further, birds with an intermediate number of MHC alleles had the largest clutch size (Bonneaud *et al.* 2004). In this study, we did not find a

correlation between parasite load and number of MHC alleles. However, we did not design our breeding program to maximise allele number diversity. Analysing only the pure lake lines to compare the results with the two laboratory studies, which also used lake offspring, number of alleles varied only from 6-9. In contrast, breeding designs by Wegner *et al.* (2003a) and Kurtz *et al.* (2004), aimed at maximizing segregation above and below optimal number of MHC alleles. This resulted in number of alleles varying from 3-9 and 2-8, respectively. In the present study, the ability to detect an effect of numbers of different alleles is clearly restricted with such a small allelic variance. Note that the average number of alleles is slightly higher in this study than those reported in previous studies (Reusch *et al.* 2001a; Wegner *et al.* 2003b; Kurtz *et al.* 2004). This is consistent with the predicted ability of the novel MHC typing system applied in this study, which uses eight primer pairs and can amplify around 20% more different MHC sequence variants than the previously published method using only two primer pairs.

In summary, we showed in a field experiment the greater importance of the genetic background compared to the MHC genotype in influencing parasite load. Despite of the demonstrated influence of MHC genes on immunity, the results presented in this paper show the importance of extending studies on the genetic basis of parasite resistance to different regions of the genetic background located beyond the MHC.

Conclusion

Given that most species on earth are parasites, understanding the implications and maintenance of genetically diverse infections are central to host-parasite evolutionary dynamics and an important challenge in biology. The main advances presented in this thesis for the field of evolutionary ecology of host-parasite interactions are threefold. First, I showed for the first time that for a parasite with a complex life cycle the genetic diversity within one host increases from the first to the second intermediate host. The genetic diversity of an infection plays an important role for sexual reproduction in the definitive host to avoid inbreeding. Thus, I propose here that maintaining the second intermediate host in the complex life cycle can be advantageous to increase the intermixture of different clones and can therefore decrease the risk of matings between genetically identical individuals in the definitive host. Second, I showed for the first time that the decrease in parasite load in mixed infections compared to single-clone infections is due to competitive suppression between co-infecting genotypes and not to a dominance effect. Third, I showed that genotype-specific host-parasite interactions arise immediately. This is the first indication in vertebrates that such specific interactions can arise independently from the MHC-mediated adaptive immune system, as the adaptive immune system needs some days to get ready.

The exact mechanisms that drive a genetically diverse infection critically influence vaccination success. On the other hand, vaccination itself alters the genetic composition of an infection. The possible consequences of such drug treatment effects on disease severity are only beginning to be investigated. Advances in predicting how the effect of drug control on genetic diversity influences total parasite load is crucial for public health, both for future disease control programs and for ongoing interventions, such as applications of strain-specific chemotherapy against malaria.

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Schools

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 1996-2001 Study of Biology at University of Basel
 Main subject: Population Biology
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 Minor subjects: Vertebrates, Invertebrates, Plant Ecology
 2001-2002 Diploma thesis at the University of Basel, Switzerland
 Group: Population biology/ S. C. Stearns, W. W. Weisser and
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 Topic: Host-parasitoid interactions
 2002-2003 One year project at the University of Jena, Germany
 Group: Population Ecology/ W. W. Weisser
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 2003-2006 PhD thesis at MPI of Limnology, Plön, Germany
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Rauch, G., Simon, J. C., Chaubet, B., Haack, L., Flatt, T. & Weisser, W. W.
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Erklärung

Hiermit versichere ich, dass diese Abhandlung, abgesehen von der Beratung durch meine akademischen Betreuer, 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. Kapitel 1, 2 und 5 wurden mit Martin Kalbe und Thorsten B. H. Reusch als Koautoren in den Zeitschriften *Molecular Ecology Notes* (Kapitel 1), *Journal of Evolutionary Biology* (Kapitel 2) und *Evolutionary Ecology Research* (Kapitel 5) veröffentlicht.

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