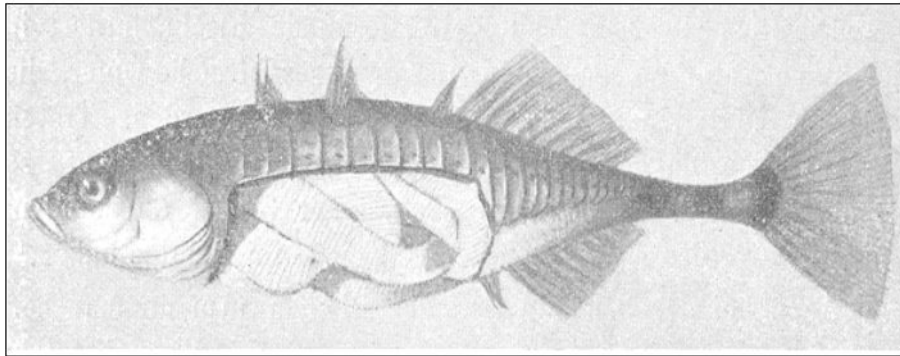


Hosts and parasites: many ways of interactions



An approach with two model organisms, the three-spined stickleback (*Gasterosteus aculeatus*) and its cestode parasite (*Schistocephalus solidus*)

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“Siehst du, Momo”, sagte er dann zum Beispiel, “es ist so: Manchmal hat man eine sehr lange Straße vor sich. Man denkt sich, die ist so schrecklich lang; das kann man niemals schaffen, denkt man.”

Er blickte eine Weile schweigend vor sich hin, dann fuhr er fort: “Und dann fängt man an, sich zu eilen. Und man eilt sich immer mehr. Jedesmal, wenn man aufblickt, sieht man, daß es gar nicht weniger wird, was noch vor einem liegt. Und man strengt sich noch mehr an, man kriegt es mit der Angst, und zum Schluß ist man ganz außer Puste und kann nicht mehr. Und die Straße liegt immer noch vor einem. So darf man es nicht machen.”

Er dachte einige Zeit nach. Dann sprach er weiter: “Man darf nie an die ganze Straße auf einmal denken, verstehst du? Man muß nur an den nächsten Schritt denken, an den nächsten Atemzug, an den nächsten Besenstrich. Und immer wieder nur an den nächsten.”

Wieder hielt er inne und überlegte, ehe er hinzufügte: “Dann macht es Freude; das ist wichtig, dann macht man seine Sache gut. Und so soll es sein.”

Und abermals nach einer langen Pause fuhr er fort: “Auf einmal merkt man, daß man Schritt für Schritt die ganze Straße gemacht hat. Man hat gar nicht gemerkt wie, und man ist nicht außer Puste.” Er nickte vor sich hin und sagte abschließend. “Das ist wichtig.”

Beppo Straßenkehrer aus “Momo” von Michael Ende

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Summary

Parasites are considered to be one of the major driving forces in the evolution of organisms. Host-parasite interactions have thus evoked great interest in evolutionary biology. However, the outcome of an infection might not only be determined by host-parasite interactions but also by within-host dynamics. Using the three-spined stickleback (*Gasterosteus aculeatus*) and the tapeworm *Schistocephalus solidus* as model organisms, various aspects of such potential interactions were investigated.

Multiple infections of the stickleback with *S. solidus* occur frequently in nature and the specific within-host dynamics might have important consequences for the outcome of the infection. Interactions between co-infecting *S. solidus* individuals could be influenced by the fact that they are likely to be future mating partners in the final bird host. In a mate choice experiment, we let cestodes choose between a related (full-sibling) and an unrelated mating partner. To investigate whether the behavioural preference is reflected in the number of eggs produced with the respective mating partner, parentage analyses of the offspring were conducted. A second experiment was carried out to calculate the difference in hatching success between related and unrelated matings. Despite the 3.5-fold lower hatching rate in incestuous matings, the cestodes showed a significant preference for their sibling. This was confirmed by the molecular data: the cestodes produced significantly more eggs with the related than with the unrelated partner (Chapter I). To investigate potential fitness consequences for *S. solidus* in multiple infections, we double infected sticklebacks. Thereby, relatedness of the parasites and the time between infections were taken into account. Double infections were established more often when the two parasites were related. In sequential infections, the secondly acquired parasite was more likely to survive and grew to a larger size compared to the first one, despite being one week younger. This difference was not apparent when both parasites were acquired simultaneously (Chapter II). An earlier *in vitro*-study suggests that *S. solidus* is capable of suppressing innate immune responses of the stickleback. However, little is known about the adaptive immune response with respect to *S. solidus* infections. By repeatedly injecting antigens *in vivo*, we challenged the adaptive part of the immune system of *S. solidus*-exposed and non-exposed sticklebacks. Parameters of innate and adaptive immunity were measured 9, 13 and 23 weeks post-infection. No adaptive immune reaction towards *S. solidus* or the applied antigens was detected. *S. solidus*

was, however, found to elicit an innate immune response confirming previous studies (Chapter III). The major histocompatibility complex (MHC) plays a key role in the specific parasite resistance of vertebrates. In the three-spined stickleback, there is mounting evidence that an intermediate rather than a maximal individual MHC allele diversity is favoured. In a correlative study, individual allele diversity of MHC class *IIB* as well as MHC class I was related to important fitness traits of the stickleback. While for male nest quality an optimum concerning MHC class *IIB* diversity was found, male breeding colouration was most intense at a maximal MHC class I diversity. Two MHC class I alleles were identified to be associated with a higher intensity of red colouration. Moreover, the probability of carrying these alleles increased with an increasing individual allele diversity, possibly explaining the positive correlation mentioned above (Chapter IV).

Zusammenfassung

Parasiten gelten als eine der treibenden Kräfte in der Evolution von Organismen. Die Interaktion von Parasiten und ihren Wirten hat daher großes Interesse in der Evolutionsbiologie hervorgerufen. Das Ergebnis einer Infektion könnte jedoch nicht nur durch Wirt-Parasit-Beziehungen, sondern auch durch Dynamiken innerhalb des Wirts bestimmt werden. Der dreistachlige Stichling (*Gasterosteus aculeatus*) und der Fischbandwurm *Schistocephalus solidus* dienen als Modellorganismen, um verschiedene Aspekte solch potenzieller Interaktionen zu untersuchen.

Mehrfachinfektionen des Stichlings mit *S. solidus* kommen häufig in der Natur vor, und die spezifischen Dynamiken innerhalb des Wirts könnten wichtige Konsequenzen im Hinblick auf das Ergebnis der Infektion haben. Interaktionen zwischen koinfizierenden *S. solidus*-Individuen könnten dadurch beeinflusst sein, dass sie mit großer Wahrscheinlichkeit zukünftige Paarungspartner im Vogelelwirt sind. In einem Wahlexperiment haben wir Parasiten zwischen einem verwandten (Vollgeschwister) und einem nicht verwandten Paarungspartner wählen lassen. Um zu untersuchen, ob sich diese Verhaltenspräferenz in der Anzahl der Eier, die mit dem jeweiligen Paarungspartner produziert wurden, widerspiegelt, wurden Vaterschaftsanalysen mit den Nachkommen durchgeführt. In einem zweiten Experiment wurde der Unterschied im Schlupferfolg von Paarungen zwischen Verwandten und Nicht-Verwandten bestimmt. Trotz des 3,5-fach schlechteren Schlupferfolgs der inzestuösen Paarungen zeigten die Parasiten eine signifikante Präferenz für ihren Verwandten. Dies wurde durch die molekularen Daten bestätigt: Die Parasiten produzierten signifikant mehr Eier mit dem verwandten als mit dem nicht verwandten Partner (Kapitel I). Um potenzielle Fitnesskonsequenzen für *S. solidus* in Mehrfachinfektionen zu untersuchen, wurden Stichlinge doppelt infiziert. Dabei wurde sowohl der Verwandtschaftsgrad als auch der Zeitraum zwischen den Infektionen berücksichtigt. Doppelinfektionen entstanden häufiger wenn die beiden Parasiten miteinander verwandt waren. In den zeitversetzten Infektionen hatte der zum späteren Zeitpunkt aufgenommene Parasit eine höhere Überlebenschance und wurde größer als der zuerst aufgenommene Parasit, obwohl er eine Woche jünger war. Dieser Unterschied trat nicht zutage, wenn beide gleichzeitig aufgenommen wurden (Kapitel II). Eine frühere *in vitro*-Studie deutet darauf hin, dass *S. solidus* in der Lage ist, Reaktionen des angeborenen Immunsystems des Stichlings zu unterdrücken. Über

das erworbene Immunsystem ist jedoch im Hinblick auf *S. solidus*-Infektionen wenig bekannt. Durch wiederholtes Injizieren von Antigenen *in vivo* wurde das Immunsystem von *S. solidus*-exponierten und naiven Stichlingen stimuliert. Sowohl Parameter des angeborenen als auch des erworbenen Immunsystems wurden 9, 13 und 23 Wochen nach der Infektion gemessen. Es wurde keine Reaktion des erworbenen Immunsystems gegenüber *S. solidus* oder der injizierten Antigene gemessen. *S. solidus* hat jedoch eine Reaktion des angeborenen Immunsystems hervorgerufen und damit frühere Untersuchungen bestätigt (Kapitel III). Der Hauptgewebekompatibilitätskomplex (major histocompatibility complex, MHC) hat eine Schlüsselrolle in der spezifischen Parasitenabwehr von Wirbeltieren. Für den dreistachligen Stichling häufen sich die Anzeichen, dass eher eine mittlere als eine maximale individuelle MHC Alleldiversität von Vorteil ist. Wir haben untersucht, ob die individuelle Alleldiversität der MHC Klassen I und IIB mit wichtigen Fitnessmerkmalen des Stichlings korrelieren. Während für die Nestqualität der Männchen ein Optimum bezüglich individueller MHC Klasse IIB-Alleldiversität gefunden wurde, war die Rotfärbung der Männchen bei einer maximalen MHC Klasse I-Alleldiversität am intensivsten. Es wurden zwei MHC Klasse I-Allele identifiziert, die mit einer intensiveren Rotfärbung in Verbindung gebracht werden konnten. Außerdem stieg die Wahrscheinlichkeit, eines dieser Allele zu besitzen mit der individuellen Alleldiversität, was möglicherweise die oben erwähnte positive Korrelation erklärt (Kapitel IV).

Introduction

Hosts and parasites

Parasitism is defined as the interaction between two species, in which the survival of one species, the parasite, is dependent on the other species, the host (MacInnes 1976). This dependence mostly involves resource exploitation that might in turn impose selective pressure on the host to develop counter-adaptations in order to avoid parasitisation. Once the host has developed adaptations to prevent parasite exploitation, the parasite might develop counter-strategies to overcome the host's resistance. Such co-evolutionary interactions potentially result in an arms race (van Valen 1973). There is no winner in this race since adaptations are followed by counter-adaptations. Inspired by Lewis Carroll's *Through The Looking Glass* (1872), these dynamics have been called Red Queen dynamics. In this tale, the Red Queen told Alice:

“Now here, you see, it takes all the running you can do, to keep in the same place”

Such an arms race does not have to be unidirectional but might result in a constant cycling of genotype frequencies in the host and parasite population (Lively 1996). A parasite genotype that easily infects a host might have a selective advantage and therefore rise in frequency imposing an increasingly higher selection pressure on the host. The host might counter-adapt and become resistant, thereby decreasing the frequency of the respective parasite genotype. By becoming resistant, this host genotype might have gained a fitness benefit and rise again in frequency (Fig. 1).

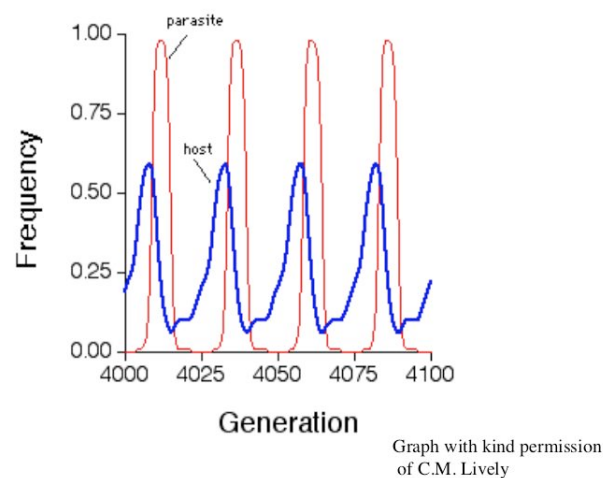


Figure 1: *Red Queen dynamics: computer simulated host-parasite co-evolution.*

Host-parasite co-evolution and the Red Queen are of great interest in the scientific world. They represent a promising hypothesis to explain the occurrence of sexual reproduction (Bell 1982; Hamilton *et al.* 1990; Ebert and Hamilton 1996), which is still an unsolved problem in evolutionary biology. Furthermore, host-parasite interactions can have important implications for the development of drugs and vaccines as well as the management of host-parasite dynamics.

The model system

The parasite's life cycle

The parasitic cestode *Schistocephalus solidus* with its complex life cycle involving three hosts (Fig. 2) represents an ideal model system to study co-evolutionary host-parasite interactions. The cestode's eggs are released into the water with the faeces of the final bird host. After two to three weeks, the first larval stage hatches. This so-called coracidium is capable of swimming actively due to a ring of cilia. In order to continue the life cycle, a coracidium has to be ingested within a few hours by the first intermediate host, a cyclopoid copepod. Inside the copepod, the larva penetrates the gut wall and establishes itself in the body cavity where it grows. This second larval stage is called the proceroid. Infectivity to the next host is reached within approximately ten days. The three-spined stickleback (*Gasterosteus aculeatus*) is the obligatory second intermediate host of *S. solidus*, and transmission occurs upon ingestion of an infected copepod. Again, the larva penetrates the gut wall and establishes itself in the body cavity where it develops into the final larval stage, the plerocercoid. Most of the growth of the cestode takes place within the stickleback host, where it reaches up to several hundreds of milligrams. The parasite is transmitted to the final host (after two to three months) when a fish-eating bird preys upon an infected three-spined stickleback. In the bird's gut, *S. solidus* becomes sexually mature and reproduction takes place. The cycle is closed when the new eggs are released with the bird's faeces, while the parent cestodes die.

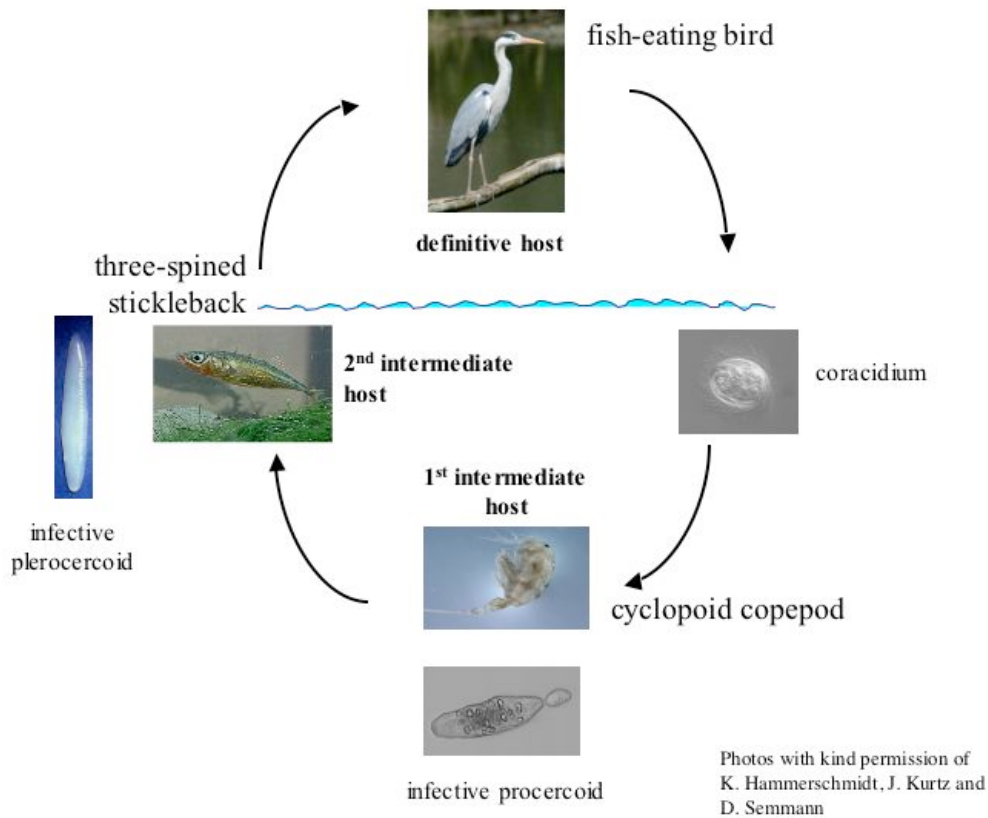


Figure 2: Life cycle of the cestode *Schistocephalus solidus*.

One big advantage of this model system is that all life cycle stages can be maintained and manipulated in the laboratory. While the two intermediate stages can easily be cultured under laboratory conditions using their natural hosts, an *in vitro* system mimicking the bird's gut replaces the final host (Smyth 1954; modified by Wedekind 1997).

One host – the three-spined stickleback

The three-spined stickleback is widely distributed in the northern hemisphere living in freshwater and brackish water, as well as marine habitats. As a visual hunter, its diet mainly consists of small invertebrates like copepods and daphnids. The three-spined stickleback is well known for its remarkable reproductive behaviour. At the beginning of the breeding cycle, males develop an intense red breeding colouration covering their throat. Once they have occupied a territory, they build a nest using plant material that is glued together with a secretion that is produced in the male's kidneys. One

characteristic of a stickleback nest is the entrance through which the female has to enter the nest to spawn the eggs. When a male detects a gravid female in his territory, he approaches her by performing a zigzag dance. If the female is receptive, showing the characteristic head-up posture, the male leads her to the nest. Once she has spawned, the male rapidly follows her and fertilises the eggs. In the three-spined stickleback, it is the male that takes care of the offspring.



Photos with kind permission of D. Semmann

Figure 3: *On the left: gravid female stickleback; on the right: male stickleback with red breeding colouration and nest*

Secondary sexual traits play a key role in mate choice. In sticklebacks, mate choice possibly includes the intensity of male breeding colouration (Milinski and Bakker 1990; Bakker and Milinski 1991; Milinski and Bakker 1992), nest quality (Barber *et al.* 2001b) and the zigzag dance (Rowland 1995). Females are expected to assess their mates by traits honestly reflecting their condition (Zahavi 1975, Hamilton and Zuk 1982). As most parasites impose costs on their host that usually reduce the host's condition, females should avoid parasitised males by assessing condition-dependent traits. Milinski and Bakker (1990) suggested that the stickleback's red breeding colouration is such an honest signal since parasitised males were not only duller, but also less often chosen by females than non-parasitised males. Considering the huge fitness costs arising from parasitisation, as well as the fact that the three-spined stickleback is host to a wide range of micro- and macro-parasites, the stickleback should have a well-adapted immune system.

As a vertebrate, the stickleback is endowed with two lines of defence, which are, however, tightly connected (Dixon and Stet 2001; Luster 2002; Goldstein 2004): the innate and the adaptive (or acquired) arm of the immune system. The innate immune system represents a quickly available, but rather unspecific, protection against pathogens (Janeway *et al.* 1999; but see Kurtz 2005) through, for instance, the respiratory burst reaction (Zapata *et al.* 1996; Whyte *et al.* 1989, Nie and Hoole 2000). During the respiratory burst, reactive oxygen molecules are generated to kill pathogens. The adaptive immune system, on the contrary, takes some time to mount the highly specific antibody-based response (Janeway *et al.* 1999). Genes of the major histocompatibility complex (MHC) play a key role in the activation of the adaptive immune system by presenting pathogen-derived peptides to T-cells that subsequently initiate antibody-production. There are two classes of MHC molecules, MHC class I and MHC class II. While MHC class I molecules bind peptides derived from intracellular pathogens, e.g. bacteria and viruses, MHC class II molecules recognise peptides from extracellular pathogens. Each MHC molecule binds only a specific set of pathogens. In the stickleback, there are probably twelve loci of MHC class I and six loci of MHC class IIB (Sato *et al.* 1998). Individual MHC diversity may thus consist of up to 24 different alleles for class I and 12 alleles for class IIB.

The three-spined stickleback thus appears to be well equipped to resist pathogen attacks.

One parasite – many ‘decisions’

Parasite-host interactions

Due to its complex life cycle, adaptations of *S. solidus* to its hosts are expected to occur at different levels. For instance, the transmission from one host to the next is essential for the completion of the life cycle, and should therefore impose a strong selective pressure. Enhanced transmission probability can, for example, be achieved by altering host behaviour such that the likelihood of the host to be preyed upon is increased. Alterations in the behaviour of parasitised hosts are well known in the literature and have been reported for a wide range of parasites (reviewed in Moore 2002). Infection with *S. solidus* has been found to induce behavioural changes in both intermediate hosts. For the copepod host, an increase in general activity and a decrease in escape ability have been described (Urdal *et al.* 1995; Jakobsen and

Wedekind, 1998). Parasitised sticklebacks swim closer to the water surface and are thus more prone to be detected by a bird predator (Giles 1987). Moreover, predation might also be facilitated because of their reaction towards predators: they show a reduced fright response to a predator attack, start foraging earlier after such an attack and swim closer to a potential predator while feeding (reviewed in Milinski 1990).

The immunological environments of the single hosts represent a second level of strong selection for parasite adaptation. Parasites might counter-adapt to an effective host immune response by either evading or fighting the immune system. One way for a parasite to evade the immune system is to migrate to a place within the host that is less, or not at all, reached by cells of the immune system. A prime example is the trematode *Diplostomum pseudospathaceum* infecting the three-spined stickleback. Once inside the fish, the parasite migrates through the body into the fish's eye lens, where it is unreachable for the immune system (Chappell *et al.* 1994). Recently, immunoevasion has also been hypothesised for helminths migrating from the gut into tissue elsewhere in the host to avoid host immune defences working at mucosal surfaces (Mulcahy *et al.* 2005). Other parasites use a more passive way and camouflage themselves to remain undetected by the host's immune system (reviewed in Damian 1997). Immunosuppression has been found in many parasite species as a way to manipulate the immune system (e.g. Boëte *et al.* 2003; Rigaud and Moret 2003; Hoerauf *et al.* 2005). Thereby, suppression might be indirect by reducing the host's general condition or direct by modulating the host's immune response. Studies on *S. solidus* suggest that this parasite has adopted multiple strategies to cope with the stickleback's immune system. *S. solidus* has been found to change its surface structure upon host switch from the first to the second intermediate host, and variability in this surface composition was related to parasite fitness (Hammerschmidt and Kurtz 2005a). This indicates that *S. solidus* masks itself to remain undetected by components of the stickleback's immune system. It also seems as if the parasite is capable of suppressing at least innate immune components: in an *in vitro* study, immune cells of *S. solidus*-infected sticklebacks failed to show an elevated respiratory burst reaction upon immune challenge (Scharsack *et al.* 2004).

Parasite-parasite interactions

As hosts are often infected with multiple parasite genotypes, being well adapted to its host might not be sufficient for a parasite. Host resources are usually limited, thus co-infecting parasites compete for these restricted resources. Competition can have large fitness consequences for the parasites since, in the worst case, it can lead to complete exclusion of the less competitive parasite (e.g. Bremermann and Pickering 1983; Nowak and May 1994). Generally, parasites face a trade-off between exploiting their host to increase their own growth and reproduction, and the risk of over-exploitation that, in the case of the host's death, would also be the end of the parasites. And competition among co-infecting parasites has been suggested to enhance the risk of over-exploitation since parasite genotypes exploiting the host's resources more rapidly are favoured (e.g. Bremermann and Pickering 1983; Frank 1992; Nowak and May 1994; van Baalen and Sabelis 1995). As predicted by kin-selection theory, competition might be reduced when co-infecting parasites are related (Hamilton 1964; Frank 1992). Related individuals share a certain amount of alleles and, by ensuring a kin's reproduction, these shared alleles will be passed on to the next generation providing an indirect fitness benefit. However, recent studies have suggested that reduced host exploitation can also exist among unrelated individuals (e.g. Chao *et al.* 2000; Brown *et al.* 2002; Schjørring and Koella 2003). In general, cooperative interactions might not only involve the common use of resources, but also sharing the costs of fighting against the host's immune system.

Multiple infections of the three-spined stickleback with the cestode *S. solidus* occur frequently in nature (Arme and Owen 1967; pers. observ.). Since transmission rates are high (Tierney and Crompton 1992), *S. solidus* individuals parasitising a single fish are likely to end up together in the gut of the final host where reproduction takes place. Hence, parasite-parasite interactions of *S. solidus* in the fish host might not only be influenced by competitive or cooperative interactions, but also by a potential mating decision.

Mating decisions

S. solidus is a simultaneous hermaphrodite, which means that each of its segments contains male and female reproductive organs; thus it is able to self- as well as cross-fertilise its eggs. Due to large fluctuations in the prevalence of *S. solidus* (Pennycuick 1971) and the fact that the time of reproduction in the bird's gut is only about ten days, the probability of a second cestode being ingested might be low. Hence, the capability of selfing ensures its reproduction when no mating partner is available. However, it also bears a cost: inbreeding depression, which is the reduction in fitness of inbred offspring in comparison to outbred offspring (e.g. Charlesworth and Charlesworth 1987). A consequence of inbreeding is a decline in genetic variability due to an increase in homozygosity. Inbreeding depression arises when deleterious mutations are accumulated because of this increase in homozygosity. *S. solidus* has been found to suffer from substantial inbreeding depression. The hatching rate of eggs produced by a selfing cestode is significantly lower than that of eggs produced by a pair (Wedekind *et al.* 1998; Christen *et al.* 2002; Schjørring 2004). Moreover, when in competition with outcrossed individuals, selfed offspring perform less well in both intermediate hosts (Christen *et al.* 2002; Christen and Milinski 2003). Despite this substantial fitness reduction when selfing, paternity analyses revealed that *S. solidus* engaged in some self-fertilisation even though a mating partner was available (Lüscher and Milinski 2003). At first sight, this seems disadvantageous because of the strong inbreeding depression. However, the benefit of preserving advantageous allele combinations may outweigh the costs of inbreeding depression (Shields 1987). Moreover, successive generations of inbreeding may lead to a decline in frequency of the deleterious alleles, thereby purging the genetic load (e.g. Charlesworth and Charlesworth 1998; Crnokrak and Barrett 2002; Glémin 2003). Thus, in the situation of ending up alone in the bird, i.e. being forced to self, inbred individuals may produce fitter offspring compared to outbred offspring.

Sexual conflict

Simultaneous hermaphrodites have not only the advantage of being able to self-fertilise, but also the disadvantage of potentially facing a sexual conflict when in an outcrossing situation. Since the fitness through eggs and sperm is expected to rarely be the same for an individual, it should take over that sexual role that has the highest pay-off (Charnov 1979). When the two mating partners prefer the same sexual role, a conflict arises, which has been called the Hermaphrodite's Dilemma (Leonard 1990). The solution to this conflict is thought to often involve reciprocity, which means that both partners take over both sexual roles (Leonard 1990; Michiels 1998). Since this is prone to cheating, i.e. one partner is not reciprocating, matings are envisioned to either happen with the two partners repeatedly alternating the sexual roles or with them continuously and simultaneously acting in both roles (Michiels 1998).

Thesis Outline

This thesis is divided into four chapters, each of which represents an independent study. Using the three-spined stickleback (*Gasterosteus aculeatus*) and its cestode parasite *Schistocephalus solidus* as model organisms, questions of parasitic mating decisions (chapter I), host-parasite interactions (chapter II and III) and host immunoecology (chapter IV) were addressed. This outline gives a short overview of the motivation for the single studies.

Chapter I

Mating decision of *Schistocephalus solidus*

Since transmission rates from the stickleback to the final bird host are high, *S. solidus* individuals co-infecting a stickleback are likely to be future mating partners. This links interactions between competitors for resources to interactions between potential mating partners. Thus, parasite-parasite interactions are likely to be influenced by the parasite's mating decision. Because of inbreeding depression, one would expect an unrelated individual to be the favoured mating partner. In a mate choice experiment, we gave cestodes the choice between a sibling and an unrelated potential mating partner. Subsequent parentage analyses of the offspring should reveal whether this behavioural preference was reflected by the actual mating preference, thus by the number of eggs produced with the respective partners when all three cestodes were bred together.

Chapter II

From the parasite's point of view

Resource limitation might lead to competition within multiple infections, which potentially results in reduced growth or even complete exclusion of the less competitive parasite. Relatedness between parasites or a temporal head-start of one of the parasites might change the outcome of the within-host dynamics. While related individuals are thought to be less competitive, an individual with a head-start might gain a competitive advantage. To investigate the within-host dynamics of multiple infections, we exposed three-spined sticklebacks to either two related or two unrelated individuals of *S. solidus* simultaneously or with seven days between the single exposures.

Chapter III

From the host's point of view

The findings presented in chapter II might support an earlier *in vitro*-study that suggested that *S. solidus* is capable of suppressing innate immune responses of the stickleback. However, little is known about the adaptive immune response with regard to *S. solidus*-infections. By repeatedly injecting antigens *in vivo*, we challenged the adaptive immune system of *S. solidus*-exposed and non-exposed sticklebacks to see whether a potential adaptive immune response in parasite-free fish was reduced in infected sticklebacks. Parameters of innate and adaptive immunity were measured 9, 13 and 23 weeks post-infection.

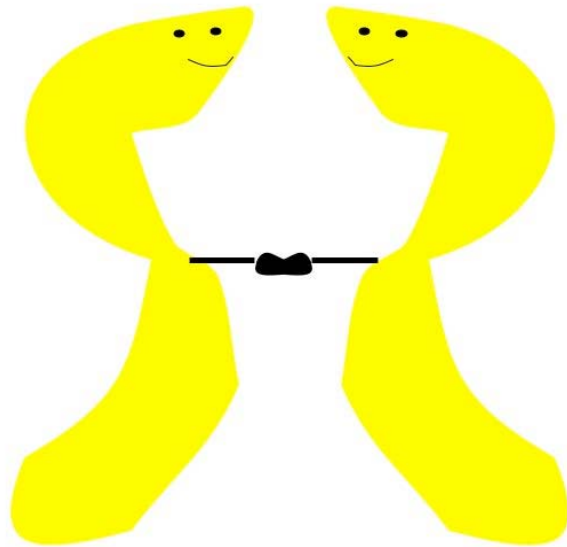
Chapter IV

Immunoecology of the three-spined stickleback

Parasites usually impose costs on their hosts and parasitisation often leads to a reduction in fitness. A good immune system, which leads to a lower parasite load, is therefore likely to be reflected in fitness related traits. Genes of the major histocompatibility complex (MHC) play a key role in adaptive immunity of vertebrates and are thus tightly linked to parasite resistance. In the three-spined stickleback, parasitisation has been shown to reduce the intensity of male breeding colouration suggesting that it reveals a male's health. Despite this close connection between the health status of a male and its breeding colouration, male redness has not been related to MHC based immunity yet. In this correlative study, we related individual allele diversity of MHC class I and MHC class IIB to male breeding colouration as well as two other important fitness traits of female and male sticklebacks, namely the number of egg clutches spawned by a female and male nest quality.

CHAPTER I

Incestuous mate preference in a simultaneous hermaphrodite with strong inbreeding depression



Abstract

Inbreeding depression and its consequences for mate choice has been extensively studied in free-living animals. However, very little is known about its significance for parasites, although it is well recognised that the mating systems of parasites can have important implications for epidemiology and parasite evolution. In this paper, we show that the cestode *Schistocephalus solidus* shows incestuous mate preference despite evidence for very strong inbreeding depression. When given the simultaneous choice between mating with a sibling and an unrelated partner, on average, the cestode showed a significant preference for the sibling cestode. To explain this surprising result, we present three hypotheses that suggest different benefits to fitness of incestuous mating, which could, alone or in concert, outweigh the cost of inbreeding depression.

Introduction

Inbreeding depression is considered one of the major selective forces for the evolution of mating systems, generally favouring matings among unrelated individuals through dispersal and kin recognition. However, while inbreeding does have detrimental effects on fitness due to the unmasking of deleterious alleles (Charlesworth and Charlesworth 1987), it is not given that breeding among relatives should always be disfavoured. The cost of inbreeding depression has to be weighed against the potential costs of inbreeding avoidance and the potential non-genetic and genetic benefits derived from breeding with related individuals. Thus, when un-related mating partners are scarce relative to related ones, the cost of inbreeding depression may be inferior to the cost of forgoing matings with related individuals (Ghiselin 1969, Waser *et al.* 1986). Furthermore, the increase in indirect fitness when breeding with kin (Parker 1979) or the benefit of maintaining locally adapted or intrinsically coadapted gene complexes (Shields 1987) may outweigh the cost of inbreeding depression. There are thus situations where one would expect a certain level of tolerance or even preference for breeding among relatives, and these indeed seem to exist (Bateson 1982, Keane *et al.* 1996, Peacock and Smith 1997, Keller and Arcese 1998, Cohen and Dearborn 2004), although they are scarce compared to studies showing inbreeding avoidance (reviewed in Pusey and Wolf 1996).

While mate choice has been extensively studied in free-living animals, very little is known about its significance for parasites, although it is well recognised that the mating systems of parasites can have important implications for clinical medicine and epidemiology (Tibayrenc *et al.* 1990; Southgate *et al.* 1995; Hastings and Wedgewood-Oppenheim 1997) as well as for the evolutionary biology of parasites, for example speciation (Southgate *et al.* 1998), host-parasite co-evolution (Galvani *et al.* 2003) and virulence evolution (Frank 1996). Molecular studies of parasite populations suggest that mating in parasites is not always random (Tibayrenc *et al.* 1990, Lüscher 2002, Razakandrainibe *et al.* 2005), and a few molecular and behavioural studies at the individual level have provided evidence from parasitic helminths for the existence of specific mate choice decisions (Tchuem Tchuente *et al.* 1995; Lüscher and Wedekind 2002).

In this paper, we test whether the cestode *Schistocephalus solidus* discriminate between related and unrelated mating partners. We do this by first studying whether

the cestode shows a behavioural preference when given the choice between a related and an unrelated potential mating partner. We then examine whether such a preference is also reflected in the number of eggs and offspring produced with the two mating partners.

S. solidus is a simultaneous hermaphrodite and can reproduce by selfing and outcrossing. However, in the studied population self-fertilised eggs hatch at a four-fold lower rate than cross-fertilised ones (Christen *et al.* 2002, Schjørring 2004). In addition, offspring of selfing cestodes perform less well in the intermediate hosts than offspring of outcrossing cestodes (Christen *et al.* 2002; Christen and Milinski 2003). Furthermore, cestodes that are left to breed alone delay the start of their egg production, presumably to wait for the arrival of a mating partner with whom they could outcross (Schjørring 2004). Such a strategy would only pay off if outcrossing is more valuable than selfing. Previous studies therefore strongly suggest that inbreeding depression plays an important role for the reproductive success of *S. solidus*. A priori, we therefore expected the cestode to show a preference for unrelated mating partners since this would likely entail the highest reproductive success. *S. solidus* is indeed capable of exerting choosiness with respect to mating partners; when given the choice, *S. solidus* spend more time close to a potential mating partner of a slightly bigger size than one of a similar or very different size (Lüscher and Wedekind 2002).

Surprisingly, we found that *S. solidus* shows a preference for close kin as mating partners despite evidence for strong inbreeding depression. We present three hypotheses that could explain this result.

Materials and Methods

Study system

Schistocephalus solidus is a trophically transmitted parasite with a complex lifecycle that involves three hosts. Growth takes place in the body cavity of the two intermediate hosts, a copepod and a three-spined stickleback (*Gasterosteus aculeatus*), while growth ceases and only reproduction takes place in the gut of the final host, a fish-eating bird. The reproductive stage lives for approximately 10 days.

The cestodes used in the experiments were the offspring of cestodes retrieved from wild singly infected three-spined sticklebacks caught in the late summer of 2000 in Northern Germany in the brackish bay of Neustadt. These wild cestodes had been

bred *in vitro* in size-matched pairs, thereby minimising the selfing rate (Lüscher and Milinski 2003). For details concerning retrieval and *in vitro*-breeding of the parent cestodes see Schjørring (2004). The eggs from 24 different clutches were hatched and the first intermediate copepod host (*Macrocyclops albidus*) exposed to single coracidia. Thirteen days after exposure, the copepods were checked for infection, and, when infected, they were fed singly to three-months old three-spined sticklebacks (for details of infection of copepods and sticklebacks, see Christen *et al.* (2002) and Christen and Milinski (2003)). These sticklebacks were the second laboratory-bred generation of sticklebacks from the Neustadt population. Approximately 3 months after infection the sticklebacks were killed by cutting their spinal cord in front of the dorsal fin and the cestodes were retrieved.

Experiments

Sixteen cestode families were randomly paired and one of the two families in a pair was randomly chosen to provide a focal cestode. For each focal cestode, two stimulus cestodes of similar size were chosen, one being a sibling of the focal cestode and one originating from a different cestode family. The three cestodes were placed in a fork-shaped nylon mesh bag of a construction similar to the one used by Lüscher and Wedekind (2002) and illustrated in their Figure 1. The mesh bag consisted of three compartments separated by seams, a central one for the focal cestode and one on each side for the stimulus cestodes. The side compartments were folded back onto the central compartment, thus allowing the focal cestode to move freely between and position itself above the stimulus cestodes. After placing the cestodes in their compartments the mesh bag was immediately sealed and positioned in a tall-rimmed glass tray filled with a medium that mimicks the gut environment of the final bird host. The tray was covered with a glass lid to minimise loss of liquid through evaporation. The related stimulus cestode was positioned to the right of the focal cestode in half of the experimental triplets and to the left in the other half. The resulting eight experimental triplets were placed in a 40°C climate chamber (simulating the body temperature of the final bird host) and illuminated only by infrared light. The cestode's gonads matures and egg production starts within approximately two days after infection of the final host (Smyth 1946), and it is within this pre-reproductive period that one would expect the cestode to be particularly active in mate choice. We thus recorded the position of the focal cestode every hour for 38 hours with a video

camera. A priori, the central compartment of the mesh bag containing the focal cestode was divided into five different sections and each assigned a number reflecting the focal cestode's distance from the sibling stimulus cestode. The section in contact with the stimulus compartment that contained the sibling cestode was assigned the value -2, the one next to this one -1, the most central part was assigned the value 0, and in symmetry the sections on the other side were assigned the values 1 and 2. Discarding recordings from the initial four hours (the focal cestodes typically did not move much within this adjustment period), we could thus calculate a mean position over the 34 hours for each focal cestode. A mean value significantly different from zero (random position with respect to the stimulus cestodes) would indicate that the focal cestode had preferred to stay close to one of the stimulus cestodes. A value significantly less than zero would reflect a preference for the related stimulus cestode, while a significantly positive value would reflect a preference for the unrelated stimulus cestode. Since the mean position is constrained to the range [-2,2] the significance was determined with a permutation test. We thus compared the obtained value with the distribution of 500 means of eight means of 34 randomly chosen position values (-2, -1, 0, 1 or 2).

To test whether an observed behavioural preference is reflected in a higher reproductive rate with the preferred stimulus cestode, we transferred the cestodes to mesh nets suspended in 40°C medium after 38 hours (see Schjørring 2004 for a detailed description). We allowed the three cestodes from each trial to interbreed for eight days. Within this period of time the cestodes produce 99% of their lifetime production of eggs (Schärer and Wedekind 1999). We then tested whether the resulting egg and offspring production complied with the behavioural preference that we had observed. We did this by hatching a sample of the eggs that a triplet had produced and assigning the parent cestodes by amplifying the five microsatellite loci SsB6, SsA22, SsA58, SsB24 (Binz *et al.* 2000) and SsCA77 (Lüscher 2002). For one of the eight triplets we could not determine which of the two stimulus cestodes was the related one and this triplet was therefore excluded from further analyses. The microsatellite technique cannot be used on eggs (Lüscher pers. comm.) and we therefore had to estimate what the offspring production corresponds to in terms of egg production. Selfing results in a lower hatching success of eggs (Wedekind *et al.* 1998; Christen *et al.* 2002, Schjørring 2004) than outcrossing, and we thus expected eggs produced with the related cestode to hatch more poorly than those produced with the

unrelated cestode. To calculate the magnitude of the difference in hatching success between related and unrelated matings, we did another experiment where we allowed cestodes to breed in pairs of sibling cestodes and unrelated cestodes. We randomly assigned cestodes from 16 families (eight of which were the same as those used in the mate choice experiments) to one of two treatments: (i) breeding in a pair with a sibling, (ii) breeding in a pair with an unrelated cestode. Cestodes in pairs were always of similar size in order to ensure a high rate of outcrossing (Lüscher and Milinski 2003). To ensure statistical independence of the statistical units, each family was represented only once in treatment (i). In treatment (ii), some families were represented several times but always with a mating partner from a different family. The cestodes were allowed to breed for eight days in the *in vitro*-system (see Schjørring 2004 for details). During the experiment, we retrieved eggs twice: after four days when the breeding medium was changed in order to ensure a constant pH level, and again when the experiment ended after eight days. The eggs were rinsed, counted and the hatching rate determined by following the procedures outlined in Schjørring (2004).

We used ANOVA models to test whether relatedness of the parent cestodes significantly affected the weight-specific egg production (Wedekind *et al.* (1998) showed that there exists a strong correlation between body size of a cestode and its egg production) or the hatching success of the eggs. Relatedness of the parent cestodes was included as a fixed factor in the models. While the hatching rate was established right away, the egg production was quantified a year after the experiment had been concluded. In eight of the samples, the eggs had clumped to a degree that precluded estimation of the number present and these samples were therefore excluded from the analysis of weight-specific egg production.

This breeding experiment allowed us to estimate the average difference in hatching rate between eggs of incestuous and non-incestuous matings. We used this to estimate the number of eggs that the focal cestodes from the mate choice experiment had produced by mating with their sib and with the unrelated cestode. We then tested whether the estimated proportion of eggs that the focal cestode produced with the two stimulus cestodes was significantly different from a 1:1 ratio with a G-test (Sokal and Rohlf 1995). Specifically, we calculated a G-value for the pooled data to test for an overall deviation of the data. In addition, we calculated a G-value that quantifies the

heterogeneity of the data to test whether the trend was the same for all the seven experimental triplets.

Finally, we tested for a significant correspondence between the behavioural mate preference and the breeding data by testing the correlation between the average position of a focal cestode and the proportion of eggs that it later produced with the related and unrelated stimulus cestodes. Since the values of both variables of the correlation are constrained, the significance of the correlation could not be determined by evaluating the ratio of explained to unexplained variance in an F-distribution. The significance was instead determined with a permutation test. For each data point the position vector was replaced by the mean of eight means of 34 randomly chosen position values (-2, -1, 0, 1 or 2). This was repeated 500 times and each time a coefficient of determination was calculated. The final distribution of coefficients of determination was then used to estimate the significance of the observed coefficient of determination.

Results

The cestodes involved in the experiment quantifying hatching success of incestuous and non-incestuous matings had a mean weight of 304.9 mg \pm 7.0 mg SE (range; 196 mg - 441 mg). While the weight-specific number of eggs was not significantly affected by the relatedness of the parent cestodes (ANOVA, $F_{1,27} = 0.31$, $P = 0.580$), eggs produced by incestuous matings had a significant 3.5-fold lower hatching success than the ones produced by outcrossing (ANOVA, $F_{1,35} = 17.56$, $P < 0.001$).

The focal cestodes involved in the mate choice experiment had a mean weight of 246.5 mg \pm 17.9 mg SE (range: 186 mg – 330 mg), while the stimulus cestodes weighed on average 252.4 mg \pm 21.2 mg SE (range: 109 mg – 379 mg). The mean position of the eight focal cestodes was -0.401 ± 0.407 SE (range: -1.76 – 1.65). On average, the focal cestode thus showed a significant preference for the side of the mesh bag that contained their sibling (Permutation test, $P < 0.002$). This behavioural preference was confirmed by the molecular data. Overall, the focal cestodes produced significantly more eggs with the related ($N = 298.5$) than with the unrelated ($N = 182$) stimulus worm (G-test, $G = 28.53$, $df = 1$, $P < 0.001$), although the ratios were not homogeneous among the cestode triplets (Heterogeneity G-test, $G = 65.96$, $df = 6$, $P < 0.001$), showing that the trend is not uniform in magnitude. Furthermore, the strength

of the behavioural preference correlated significantly with the relative proportion of the focal cestode's eggs that were produced by mating with the two stimulus cestodes (Permutation test, $P < 0.02$, Fig. 1). Thus, the stronger the preference shown by a focal cestode for a specific stimulus cestode, the larger the proportion of eggs that it produced with this stimulus cestode.

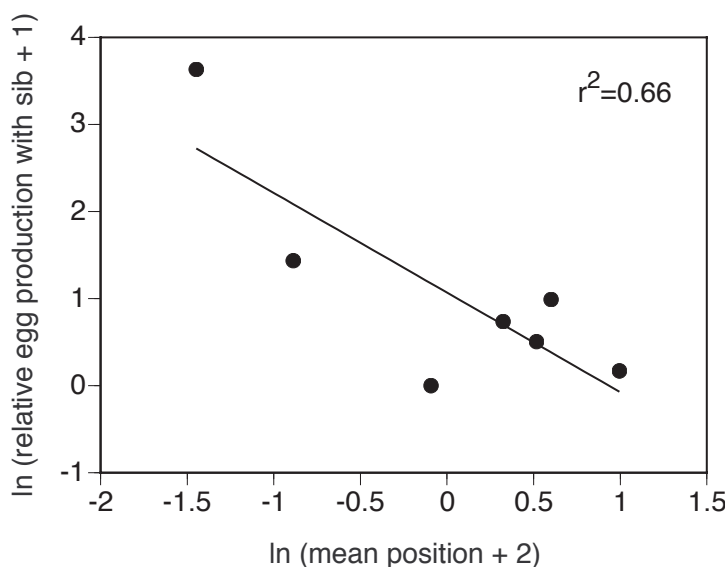


Figure 1: Correlation of the behavioural and reproductive mate preference of a focal cestode exposed to a sibling and an unrelated stimulus cestode. The abscissa shows the \ln -transformed mean position of a focal cestode over a period of 34 hours, low values being a preference for area close to the sibling. The ordinate shows the \ln -transformed estimated egg production by a focal cestode with its sibling relative to its production with the unrelated cestode.

Discussion

Cestodes that reproduced with a sibling experienced a 3.5-fold lower hatching success of their eggs than cestodes that had an unrelated mating partner. This result confirms earlier findings in this species showing that selfing implies a considerable decrease in hatching success (Wedekind *et al.* 1998; Christen *et al.* 2002, Schjørring 2004). Considering these detrimental effects of incestuous matings one could expect that evolution would favour an avoidance of such matings when alternatives are present. However, when cestodes were given the simultaneous choice between an unrelated and a sibling cestode, they showed a strong preference for the sibling cestode. During

the pre-breeding period, they spent significantly more time close to the sibling cestode than to the unrelated cestode. In addition, when allowed to breed, they produced significantly more eggs with the sibling cestode than with the unrelated cestode. The result is all the more surprising considering the cestode's parasitic lifestyle, which is expected to cause it to be under strong selection to maintain a high evolutionary potential to counteract the evolution in the host of new mechanisms to avoid or kill it (Galvani *et al.* 2003).

The overall clear preference for kin notwithstanding, the variation among individuals was substantial. Thus, while some focal cestodes showed a very strong preference for their sibling as a mating partner, others showed a more modest preference (Fig. 1). It is possible that this variation reflects a variation in the cost of breeding with relatives. Lüscher (2002) found that, when provided with a mating partner, cestodes whose selfed offspring had a high hatching rate produced relatively less by outcrossing and more by selfing than those whose selfed eggs had a low hatching success. It is therefore conceivable that the focal cestodes that showed a high preference for kin in our experiment were the ones that would experience the lowest cost of inbreeding in terms of a lower hatching success, and vice versa for those that showed a more modest preference. Unfortunately, the small number of successful infections did not allow us to have all the families from the mate choice experiments represented in the experiment quantifying hatching success of incestuous and non-incestuous matings. Only two of the families that provided focal cestodes were represented in the hatching experiment as sib mating. However, it is unlikely that a variation in hatching success would change our results qualitatively. Indeed, the 'real' slope of the correlation between attraction and egg production would be steeper than the one we found.

However, although there may be variation in the extent to which individuals suffer from inbreeding depression, a substantial benefit of breeding with kin that could outweigh the cost of inbreeding depression remains to be identified in order to explain the significant overall preference for mating with close kin.

Firstly, it is possible that the kin-selected benefit of mating incestuously, i.e. the increase in indirect fitness, is large enough to outweigh the decrease in direct fitness due to inbreeding depression (Parker 1979). In a recent paper Kokko and Ots (2006) showed that, in theory, a very high inbreeding tolerance may evolve in species with low parental investment, which, in addition, experience a low mate encounter rate, especially when these encounters are sequential rather than simultaneous. *S. solidus*

indeed has low parental investment; once mature, it produces and releases its eggs continuously into the gut of its final bird host throughout its short reproductive life. Furthermore, mating events are likely to follow each other with little or no delay and may even be simultaneous (pers. obs.). Our experimental design forced the cestodes to perform simultaneous mate choice, a scenario that favours relatively low inbreeding tolerance. However, given the short reproductive life span of the cestode (10 days), it is possible that mate choice under natural conditions, where the trait has evolved, is sequential rather than simultaneous, in which case a higher level of inbreeding tolerance would be expected. It would be interesting to study the mate preference of the cestode when exposed sequentially to related and unrelated mating partners.

Secondly, there may be direct fitness benefits associated with incestuous breeding, which could outweigh the cost of inbreeding. As a simultaneous hermaphrodite, *S. solidus* plays both the female and the male role during mating. Fertilisation is internal and sperm is transferred by penis insertion. The expected fitness through eggs and sperm will rarely be the same for an individual and it should prefer the one of the two sexual roles, which has the highest expected payoff (Charnov 1979). When two mating partners prefer the same sexual role, a conflict is likely to ensue. The solution to this conflict is thought to often involve conditional reciprocity where an individual's willingness to play the unpreferred sexual role depends on the partners willingness to, in turn, reciprocate such an act (Michiels 1998). Mating is thus envisioned as either repeated alternations of sexual roles by the two partners or a continuous and simultaneous act in both sexual roles by the two partners. The main point with such a strategy is that it allows an individual to abandon a mating, without too great a loss, if the partner does not reciprocate. A requirement for this strategy to evolve is that it should not be too costly to leave a partner that does not reciprocate, for example in terms of finding a new mating partner (Michiels 1998). This requirement is likely to be fulfilled for *S. solidus*. The final bird hosts are capable of consuming a considerable number of sticklebacks (the second intermediate host) every day (Kniprath 1965) so even with a moderate prevalence in the fish population the cestode is likely to encounter several potential mating partners in its lifetime. Alternatively, it could opt for fertilising itself. If two potential mating partners are related, then the indirect fitness at stake should increase their mutual interest in solving the conflict of interest with respect to sexual role and both should be more

willing to take on the unpreferred sexual role. With a lower risk of cheating, the mating partners can invest less in controlling each other and reproduction may be more time efficient; sperm exchange may be faster if the mating partners can trust each other. This could be of particular importance if mature individuals face a considerable mortality risk and time therefore constitutes an important constraint on their reproductive success. Under these circumstances, the higher efficiency of incestuous matings may outweigh the cost in terms of inbreeding depression and select for an incestuous mate preference. In the experiment comparing the reproductive success of sib-mating and outcrossing cestodes, we found no difference in the amount of eggs produced. However, there is no extrinsic source of mortality in our experiment and by removing the time constraint from the system we may have covered up an initial difference in the efficiency of egg production. It would therefore be interesting to test this idea by studying the phenology of egg production during sib-matings and unrelated matings.

Finally, incestuous mating could be favoured by selection at the population level if populations where individuals regularly inbreed are less likely to go extinct than populations where inbreeding is avoided. Maternally inherited cytoplasmic genetic elements with deleterious effects on male fitness could give rise to such a scenario. Engelstädter and Charlat (2006) recently suggested that this kind of genetic elements can spread in outbreeding populations. This is because males hosting the deleterious cytoplasmic element mate more often than at random with unrelated females that do not host the element, thereby reducing their average fitness and increasing its own indirect fitness through its sisters (Hamilton 1970). At least in theory, the invasion of a deleterious cytoplasmic element can lead to the population's extinction due to the overall lower offspring production (Engelstädter and Charlat 2006). Populations where individuals regularly inbreed may be inherently protected from the invasion of deleterious cytoplasmic elements since such elements would be selected against: males hosting a deleterious cytoplasmic element would lower the fitness of related females (which also host the element) relative to that of unrelated females. Inbreeding populations could thus be maintained because their efficient purging of deleterious cytoplasmic elements increase their average offspring production and lower their extinction risk relative to that of outbreeding populations.

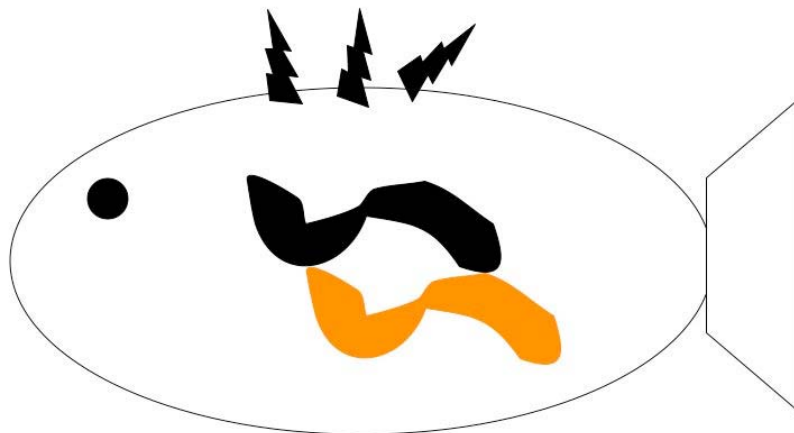
The three hypotheses are not mutually exclusive and could act in concert.

The population from which the cestodes of this study originate has previously been found to show a significant heterozygote deficiency (Lüscher 2002). This was interpreted as being due to regular selfing by the cestodes. Our results offer an additional explanation for the low genetic variability in the cestode population since regular breeding with kin would contribute to a deprivation of the genetic variability in the cestode population.

In conclusion, we found that eggs from sib matings of the cestode *Schistocephalus solidus* had a 3.5-fold lower hatching success than eggs from matings of unrelated cestodes. Despite this very high cost of incestuous mating, when given a choice between mating with a sibling or an unrelated partner, on average, the cestode showed a significant preference for the sibling cestode. However, there was considerable individual variation in mating preference. Apart from suggesting that *S. solidus* is capable of kin recognition and active mate choice, the results thus challenge our naïve expectations concerning the selective pressures involved in the evolution of mating behaviour in this cestode.

CHAPTER II

**Multiple infections: Relatedness and time
between infections affect the establishment and
growth of the cestode *Schistocephalus solidus* in
its stickleback host**



Abstract

We studied experimental double infections of the cestode *Schistocephalus solidus* in its stickleback host. In particular, we were interested in how two important components of the cestode's transmission success - establishment and growth within the fish host - were affected by the relatedness of the two parasites in a double exposure and by the timing of the two exposures, i.e. whether they occurred simultaneously or sequentially. We found that male sticklebacks more often became infected (singly or doubly) if the two cestodes in the exposures were related, while female sticklebacks were more easily infected (singly or doubly) when exposed to two unrelated cestodes. Irrespective of the fish's gender, successful infections more often contained both cestodes when they were related. In sequential exposures with related as well as unrelated cestodes, the cestode in the later exposure survived better and also grew larger than the cestode from the first exposure, despite being one week younger. Our results emphasize that within-host dynamics and factors acting at this level can play an important role in determining a parasite's transmission success.

Introduction

Infections of hosts by multiple strains or individuals of parasites occur in many host-parasite systems. Recent theoretical studies have emphasized that details of the within-host dynamics of multiple infections can have a large influence on the evolution of the parasite's life-history strategy, e.g. its virulence (van Baalen and Sabelis 1995; Chao *et al.* 2000; Brown *et al.* 2002; Schjørring and Koella 2003). Indeed, multiple infections can lead to either higher or lower virulence, depending on the specific dynamics within the host and their effect on parasite fitness (such as cooperative interactions among parasites or a trade-off between fecundity and competitive ability of the parasite). Such studies therefore stress the importance of relatively detailed knowledge of the within-host dynamics and how they affect parasite fitness for qualified predictions about virulence evolution. However, studies that offer this kind of information are still rare and more are needed if we wish to reach a deeper understanding of evolutionary epidemiology.

In this paper, we address the effect of multiplicity of infection on a parasite's transmission success by focusing on two factors of potential importance for within-host dynamics: (i) relatedness (or genetic variation) within multiple infections, and (ii) the time between multiple acquisitions of parasites.

While relatedness was originally suggested to reduce competition among co-infecting parasites (Frank 1992), recent studies have argued that relatedness can also sometimes intensify competition to a level where it outweighs any benefits of altruism (Queller 1994; West *et al.* 2001; Griffin and West 2002). High levels of competition may result in larger size differences among co-infective parasites (Christen *et al.* 2002; Puustinen *et al.* 2004) or even complete exclusion of the less competitive parasite (Bremermann and Pickering 1983; Nowak and May 1994; Christen *et al.* 2002; Wille *et al.* 2002). In addition, genotype-specific interactions with the host's immune system can amplify or invert inherent differences in competitive ability of different parasite genotypes (Read and Taylor 2001). This could, for example, result from a high growth rate attracting the attention of the immune system (de Roode *et al.* 2004), or from a trade-off between the parasite's growth rate and its ability to suppress the host's immune system (Cox 2001; Hughes and Boomsma 2004).

In addition to the relatedness of parasites in a multiple infection, the timing of the acquisition of the multiple parasites, i.e. whether infections are simultaneous or

sequential, may influence the within-host dynamics and thus the outcome of the interaction in terms of the parasites' transmission success. The naive expectation is that the parasite that establishes itself first in the host should have a competitive advantage over parasites that arrive later because of its head-start with respect to exploitation of the host's resources or because the host's immune system has become stimulated by the first parasite. There are indeed data in support of this scenario (Read and Taylor 2001; Hood 2003). However, due to the interaction with the host's immune system, it is not clear that it should always be an advantage to be the first parasite to infect a host. The first parasite may have to pay a relatively higher cost of being the sole target of the immune defense, or the sole provider of immunosuppressive substances. Alternatively, the penetration of the host by a parasite may stimulate a part of the immune system that is different from the part engaged in fighting a parasite once it has settled in the host (Hammerschmidt and Kurtz 2005b), or the defense may be compartmentalized within the host so that immune-active cells and products are concentrated in the region of the body where the parasite is found (Janeway *et al.* 1999). If there exists, within a host, a trade-off between investment in those different functional or spatial parts of the immune defense, then an already established parasite that is keeping one part of the immune system busy, causing less resources to be available for the other part, could facilitate the entrance of a second parasite.

In order to study how a parasite's transmission success is affected by the multiplicity of infection, and in particular by the relatedness of the multiple infection and the timing with which multiple parasites are acquired, we used the cestode *Schistocephalus solidus* (Müller 1776) and its second intermediate host, the three-spined stickleback *Gasterosteus aculeatus* (Linnaeus 1758) as a model system. Specifically, we experimentally investigated the consequences of double infections, their relatedness and temporal foundation for two important components of the cestode's transmission success: establishment and growth within the stickleback host. The size obtained by the cestode in the fish host is a good proxy of the parasite's expected fecundity since there is a strong positive correlation between the body size of the cestode when it is transmitted to the final host and its total egg production (Wedekind *et al.* 1998). We found that relatedness of the cestodes as well as the time between infections had an effect on their establishment and growth. Male and female sticklebacks differed in their susceptibility to related and unrelated exposures.

Furthermore, in successful infections, both cestodes were more likely to become established if they were related. We also report the surprising finding that, in sequential infections, the cestode acquired last survived better and grew larger than the cestode acquired first, despite being one week younger.

Materials and Methods

Study system

The cestode *Schistocephalus solidus* is a trophically transmitted parasite with a complex life cycle involving three hosts. Eggs are excreted into freshwater with the feces from the final bird host. After hatching the cestode larva is ingested by a cyclopoid copepod and grows for approximately two weeks in the body cavity of this host before being transmitted to the second intermediate host, the three-spined stickleback (*Gasterosteus aculeatus*), upon ingestion. The cestode larva penetrates the gut wall of the fish and settles in the body cavity, where it continues to grow for several weeks to several months until it is transmitted to its final host when a bird eats the infected fish. The cestode only lives for a few weeks in its final host, where its growth ceases and only maturation and reproduction take place.

The fish and the parasites used in this experiment were the second laboratory-bred generation of fish caught in the Binnensee in Neustadt (Northern Germany) in autumn 2001. Infected copepods are exceedingly rare and multiply infected ones have never been observed in this location (pers. obs.). However, double infections of *S. solidus* are regularly found in this population of three-spined sticklebacks. In a study during the previous year 9% ($N = 195$) - 12% ($N = 178$) of the infected fish were doubly infected (pers. obs.).

Experimental procedure

Offspring from a first generation of randomly paired wild fish were mated to create the three genetically independent fish families used in the experiment (see Bakker (1986) for details of breeding sticklebacks in the laboratory). At the time of infection the fish were between two and five months old.

The cestodes were bred using an *in vitro* technique developed by Smyth (1954) and modified by Wedekind (1997). *S. solidus* is a simultaneous hermaphrodite that can reproduce by self- and cross-fertilisation. However, we ensured a high rate of

outcrossing by pairing only cestodes of similar size (Lüscher and Milinski 2003). We used the F2 generation of the wild cestodes to obtain nine sib-families. We split the nine families into three groups of three families, of which two shared a set of grandparents (and the individuals were thus cousins) and one shared no parent or grandparent (and was thus considered unrelated). None of the families shared a parent or a grandparent with a family from another group. From each group, or triplet, we chose one of the related families to provide focal cestodes (F), while the other one provided related cestodes (R) of the focal cestode. The last family provided unrelated cestodes (U) to the experiment.

We exposed copepods (*Macrocyclops albidus* (Jurine 1820)) to individual, newly hatched coracidium larvae (see Christen *et al.* (2002) for a detailed description of the method). Successfully infected copepods were selected 10 days after infection to infect fish on day 11. To increase the probability of copepod consumption, the experimental fish were starved for two days before exposure. Each fish was transferred to a 1 l aquarium in the evening, and the following morning, the infected copepods were added to the aquaria. After 24 hours, we transferred each fish to one half of a 16 l aquarium. Each 16 l aquarium was divided by a grey opaque partition that was attached to the tank with silicon. The water from the small aquaria was filtered in order to detect uneaten copepods. Overall, only one uneaten copepod was found.

We exposed the three stickleback families to five different infection treatments, each family with a different parasite triplet: (I) singly exposed to F, (II) doubly exposed to R and F simultaneously, (III) doubly exposed to U and F simultaneously, (IV) singly exposed to R and then F seven days later, (V) singly exposed to U and then F seven days later. We thus deliberately confounded fish family and cestode triplet (creating what we call ‘family groups’), thereby avoiding effects of possible genotype-genotype interactions. The delay of one week between sequential exposures allowed the first parasite enough time to penetrate the gut wall of the fish and settle in the body cavity before the second parasite was ingested (Hammerschmidt and Kurtz 2005b).

Each treatment was replicated between 6 and 16 times per stickleback family; the number of replicates depended on the number of infected copepods. Within each family, fish were randomly assigned to the different treatments, with the constraint that the mean fish length was similar for the five treatment groups. In order to control for the fact that the two exposures in sequential double infections took place on

different days, we performed the single exposures and simultaneous double exposures on different days as well. We pooled the data from the different experimental days before analysis. All experimental fish were handled in the same way, i.e. they were all caught twice with an interval of seven days and kept in the 1 l aquaria for the same amount of time. Doubly exposed fish received a total of two infected copepods while singly exposed ones received one infected copepod.

During the experiment, the fish were kept singly at 18°C. Within the room, distribution of the treatments was randomized. The fish were fed three times a week with a defined amount of frozen chironomid larvae (1 ml, measured in a glass pipette; reduced to 0.5 ml during the experiment). To prevent them from feeding on algae they were transferred to clean aquaria every 2-3 weeks.

Dissections took place 13 weeks after exposure to the focal parasite F. The fish were killed by a cerebrospinal cut. For each fish, weight (to the nearest mg), body length (from the snout to the base of the tail, to the nearest mm) and gender was determined. When infected, the parasites were removed from the body cavity of the fish with a pair of blunt forceps, weighed (to the nearest mg) and conserved in alcohol (70%) for further genetic analysis. Each parasite could be identified unambiguously by microsatellite analysis, for which we used two microsatellite loci (SsCTA22 and SsCAB6; Binz *et al.* 2000).

Statistical Analysis

We tested for a difference in mean initial fish size among family groups and among treatments within family groups by performing an analysis of variance (ANOVA), including family group and treatment as independent factors. The family groups turned out to differ in the mean initial length of the fish. A potential effect of the family group is therefore partly confounded by initial fish size. Although independent variables in statistical tests should not be confounded, we nevertheless included initial fish size in the analyses because it is known to have an effect on the size of the cestode (Schärer and Wedekind 2001; Lüscher and Milinski 2003). Therefore, for each of the following analyses, all of which included family group and initial fish size as independent variables, we first performed our statistical tests on a reduced data set, from which the fish that caused this redundancy of the two variables were excluded. If neither family nor initial fish size interacted with the other independent variables (which always turned out to be the case), the analysis was then performed on the full

data set, including family group and initial fish length as factor and covariate respectively.

Unless otherwise stated, all the following analyses on doubly exposed fish included, in addition to family group and initial length of the fish, the gender of the fish as well as the relatedness of the parasites and the time between the exposures as factors. All possible interactions were initially included in the model and insignificant ones subsequently removed by performing backward elimination (removal at the 10% significance level). We used logistic regressions to test for significant effects on the success of infection and on the intensity of the successful infections. An infection was defined as being successful if at least one cestode had become established in the fish. Performing the same kind of analysis, we then examined whether we could identify significant effects on the probability for the focal cestode to be the one surviving in the doubly exposed fish that turned out to be singly infected. Since the effect of the time between exposures was almost significant in this analysis ($P = 0.073$) we then used G-tests (Sokal and Rohlf 1995) to determine whether one or both infection regimes gave rise to a survival probability significantly different from 0.5 (the survival probability expected if it is random which of the two cestodes survives).

To test for significant effects on the growth of the focal cestode, we used an analysis of variance. We performed the test for the focal cestodes that were found to share the host as well as for those found to inhabit the host alone at the time of dissection. In order to elucidate whether there was a difference in weight between the two cestodes within the same double infection, we performed paired t-tests of the focal and non-focal cestodes' weight. We did this separately for simultaneous and sequential double infections.

Finally, we used analyses of variance to compare the weight attained by the focal cestode in singly and doubly exposed fish. The previous tests revealed that the relatedness of a double exposure did not influence the weight attained by the focal cestode, and we therefore did not consider this variable in the current analyses. We performed four separate comparisons, distinguishing focal cestodes from simultaneous and sequential exposures that turned out to be single or double infections. We included family group, fish gender and initial fish length as additional independent variables in these ANOVA models.

For all statistical analyses, we used the software JMP 5.0.1.2 for Macintosh (SAS Institute Inc. 2003).

Results

We exposed a total of 193 fish, of which 10 died during the experiment. The dead fish were all infected at the time of death and nine of them had been doubly exposed, eight of them sequentially. They were all excluded from further analyses. Of the fish that survived, 156 were doubly exposed and 27 singly exposed to the cestode. The numbers of fish in the three family groups and their mean initial lengths were 74 of length 3.60 cm \pm 0.02 cm SE (range 3.4 - 3.9 cm), 46 of length 3.55 cm \pm 0.02 cm SE (range 3.2 - 3.9 cm) and 63 of length 3.38 cm \pm 0.03 cm SE (range 3.0 - 4.1 cm). An ANOVA including treatment and family group as factors revealed that, while there was no difference in the mean initial fish size among the five treatments within families (ANOVA, $F_{4,176} = 0.46$, $P = 0.763$), one of the families was significantly smaller than the others (ANOVA, $F_{2,176} = 1.78$, $P < 0.001$). We therefore controlled for this difference when choosing the statistical models used for the analyses by proceeding as described in the method section.

44.4% of the singly exposed fish ($N = 27$) were infected 13 weeks later and the cestodes had grown to a mean weight of 182.2 mg \pm 8.2 mg SE. The probability that at least one cestode became established when the fish had been exposed to two cestodes was significantly affected by an interaction between the gender of the fish and the relatedness of the two cestodes (Table 1A). While females were 8% more likely to become infected (singly or doubly) when exposed to unrelated cestodes, males were 14% more likely to become infected (singly or doubly) when exposed to related cestodes (Fig. 1). The relatedness of the exposure also had a significant effect on whether both or only one of the cestodes became established in the successful infections (Table 1B). The two cestodes were 22% more likely to both become established if they were related (Fig. 1).

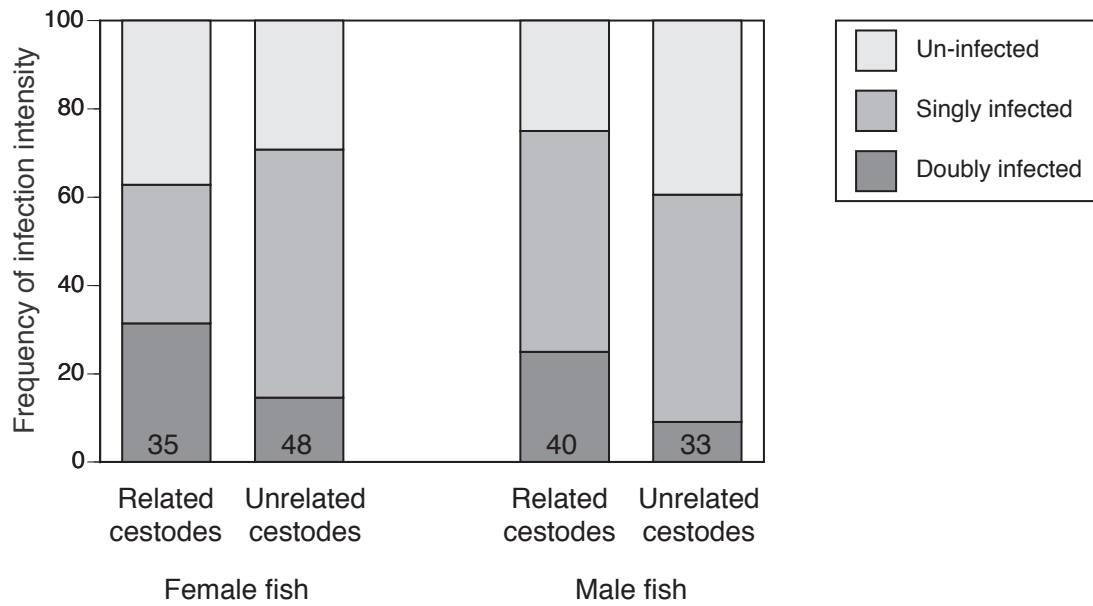


Figure 1: Distribution of infection intensities of the cestode *S. solidus* in male and female sticklebacks exposed to two related or unrelated cestodes. Dissections took place 13 weeks after exposure of the fish to the focal cestode. Sample sizes are given at the bottom of the bars.

In those doubly exposed fish that ended up being singly infected, there was an almost significant effect of time between exposures on the probability that the focal cestode would be the one inhabiting the host (Table 1C). This trend was due to the fact that the two cestodes in double exposures had the same probability of being the one left in simultaneous infections (G-test, survival probability for focal cestode: 52.6%, $N = 38$, $df = 1$, $G = 0.105$, $0.5 < P < 0.9$, Fig. 2), while the second exposure (the focal cestode) was significantly more likely to end up alone in the host in sequential infections (G-test, survival probability for focal cestode: 73.0%, $N = 37$, $df = 1$, $G = 8.112$, $0.001 < P < 0.005$, Fig. 2). In addition to this higher probability of surviving in the host when being the second exposure of a sequential double infection, these late arrivals also grew to a significantly larger size (Table 1D). After 13 weeks in the fish host, focal cestodes from sequential double infections were 19% heavier than those from simultaneous double infections (Fig. 3C). A similar effect, although not significant (ANOVA, $N = 47$, $SS = 2405.8$, $F_{1,40} = 3.07$, $P = 0.088$), was evident for focal cestodes left as single infections in doubly exposed fish (Fig. 3B); focal cestodes from fish exposed sequentially were 7% heavier than those from fish exposed simultaneously. Comparing the weights of the cestodes within the same double

infection revealed that while the focal and non-focal cestode had similar weights in simultaneous infections (paired t-test, $N = 16$, $df = 15$, $t = 0.30$, $P = 0.768$, Fig. 3C), the focal cestode was 17% heavier than the non-focal cestode in sequential infections, despite being one week younger (paired t-test, $N = 15$, $df = 14$, $t = 2.52$, $P = 0.024$, Fig. 3C).

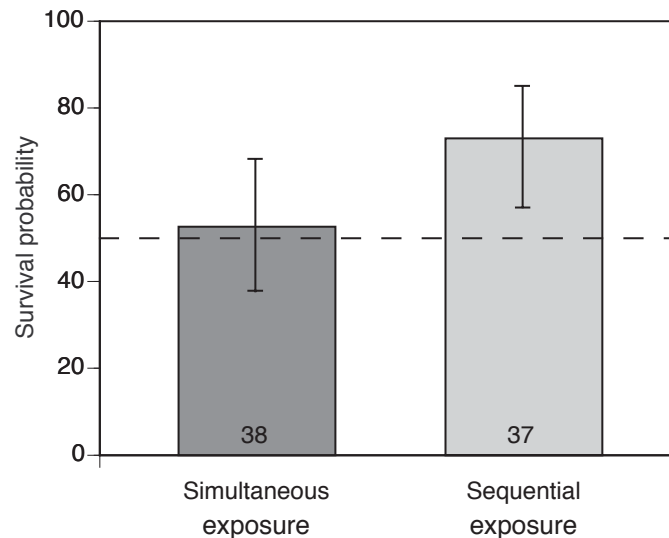


Figure 2: Frequency with which the focal cestode *S. solidus* was the one left in doubly exposed but singly infected sticklebacks 13 weeks after the fish was exposed to the infective stage of the focal cestode. The focal cestode infected the stickleback one week later than the non-focal cestode in sequential infections. The dashed line indicates the 50 % frequency, i.e. the frequency expected when it is random, which of the two cestodes is left. Sample sizes are given at the bottom of the bars. Lines above and below the bars indicate the 95 % confidence intervals.

Focal cestodes from doubly exposed but singly infected fish did not differ in weight from those from singly exposed fish (Fig. 3B). However, on average, focal cestodes from simultaneous and sequential double infections weighed 32% and 19% less, respectively, than focal cestodes from singly exposed fish (Fig. 3C).

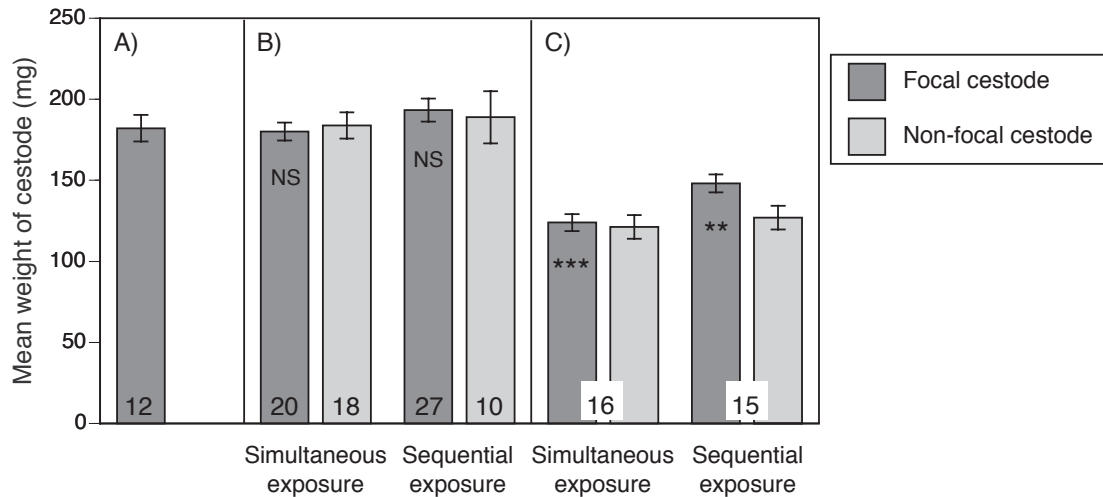


Figure 3: Mean weight of the cestode *S. solidus* 13 weeks after exposure of the fish host to the focal cestode. Weights of unrelated and related non-focal cestodes are pooled. A) Cestodes from singly exposed, singly infected fish, B) cestodes from doubly exposed, singly infected fish, C) cestodes from doubly exposed, doubly infected fish. Sample sizes are given at the bottom of the bars. Lines above and below bars indicate one standard error. Within the bars for the focal cestodes from doubly exposed fish (B and C) the significance level of the difference to the mean weight of singly exposed cestodes (A) is indicated (***: $P < 0.0001$, **: $P < 0.001$, NS: $P > 0.05$). The probability was estimated with an ANOVA model including family group, initial fish length and fish gender. Note that the non-focal cestodes in C are one week older than the rest.

Independent variables	df	A		B		C		D		
		Infection success		Infection intensity		Survival of focal cestode		Weight of focal cestode		
		(N = 156)		(N = 106)		(N = 75)		(N = 31)		
		χ^2	P	χ^2	P	χ^2	P	SS	F	P
Family	2	13.62	0.001	1.49	0.475	1.01	0.605	5684.7	9.93	0.001
Initial fish length	1	0.24	0.627	0.68	0.410	0.02	0.882	219.1	0.77	0.390
Fish gender	1	0.10	0.746	0.46	0.497	0.60	0.440	64.7	0.23	0.639
Relatedness of cestodes	1	0.16	0.690	7.11	0.008	0.02	0.882	66.3	0.23	0.635
Time lag of infection	1	0.53	0.466	0.00	0.945	3.23	0.073	4257.3	14.87	0.001
Gender*relatedness	1	4.62	0.032							
Error (ANOVA)	24							6871.6		

Table 1: Effects of the listed independent variables on the establishment and growth of the cestode *S. solidus* in doubly exposed sticklebacks. Success (A) and intensity (B) of infection, in addition to the survival probability of the focal cestode in singly infected sticklebacks (C) were tested with logistic regressions (using log-likelihood ratios to obtain the chi-square values), while an analysis of variance was used to test the weight of the focal cestode in doubly infected fish (D). Dissections took place 13 weeks after the fish had been exposed to the focal cestode. Only successful infections (i.e. where at least one cestode became established) were considered for the analysis of infection intensity.

Discussion

We found that both relatedness of double exposures and a time lag between exposures of the cestode *S. solidus* affected two important components of its transmission success: establishment and growth within its stickleback host.

While the probability that at least one cestode became established was higher for two related cestodes than for two unrelated ones in male sticklebacks, the opposite was true for female sticklebacks; they were more easily infected by at least one cestode when exposed to two unrelated cestodes than by two related ones (Table 1A, Fig. 1). This gender difference could reflect that males cope better with a variable sample of smaller amounts of antigens, while females are more powerful in fighting larger amounts of less variable antigens. Our result thus suggests that males and females may differ in the way resources are distributed among different immune functions. Many studies have shown evidence for a gender-biased susceptibility to parasites (reviewed in Klein 2000). This bias has been related to gender-specific differences in resource allocation to reproduction and immune function (Folstad and Karter 1992; Poulin 1996a; Wedekind and Jakobsen 1998) as well as in the exposure to pathogens (Tinsley 1989; Reimchen and Nosil 2001). However, a difference in susceptibility to infection has not previously been linked to the genetic variability of the exposure.

In both male and female sticklebacks, relatedness of double exposures resulted in a higher intensity of infection: successful infection more often resulted in a double infection if the two exposures were related (Table 1B, Fig. 1). It is possible that once the cestodes become established within the host, two related cestodes with similar antigens simply overwhelm the immune system of the fish. Alternatively, the cestodes may be more effective at suppressing the host's immune system if they are related, either because they, as kin, are more likely to cooperate and share the cost (Hamilton 1964; Frank 1992; Brown 1999), or because an effective genotype-specific suppression requires less of a contribution from each individual (Brown *et al.* 2002). Finally, kin-selection may favour less intense competition among related cestodes for the host's resources (Hamilton 1964; Frank 1992). Our findings are consistent with the results of Wedekind and Rüetschi (2000) who exposed individuals of the first intermediate host, the copepod *Macrocyclus albidus*, to multiple infections of *S. solidus*. They found that exposing the copepods to six cestodes from a mix of two different clutches of *S. solidus* (high genetic heterogeneity) resulted in fewer multiple

infections than exposing them to six cestodes from only one clutch (low genetic heterogeneity). In addition, they found that the copepods were more likely to become infected when they were exposed to a mix of clutches than when exposed to only one clutch.

Not only the relatedness to the other cestode in a double exposure but also the timing of the exposures strongly affected the infection success of an individual parasite. In simultaneous infections, both parasites had equal chances of successfully becoming established in doubly exposed but only singly infected fish (Fig. 2). More surprisingly, though, the cestode of the second exposure of sequential infections was more likely to survive than the one of the first exposure (Fig. 2). In addition, in doubly infected fish, the secondly acquired focal cestode grew to a significantly larger size, not only when compared to focal cestodes of similar age in simultaneous double infections (Table 1D), but also when compared to its one week older partner within the same host (Fig. 3C). This size difference was also evident, although not significant, in doubly exposed but singly infected fish when comparing focal cestodes from second exposures to non-focal cestodes from first exposures (Fig. 3B). That the effect here is less strong than in the doubly infected fish is perhaps not surprising. We do not know at what stage the second cestode failed to become established in the host, but it is likely that some of the double exposures were essentially single exposures. Thus, including these in the analysis would weaken an effect.

The surprising finding that there is an advantage of being the later cestode of a sequential infection is contrary to the results of the few other studies of sequential infections (Read and Taylor 2001; Hood 2003), where the first infection was always found to have the competitive advantage. Our result is not likely to be due to the later cestode simply being competitively superior to the first one since the same cestodes grew to similar sizes when they were in simultaneous infections (Fig. 3C). Rather, our finding suggests that there is a lower cost associated with being the later cestode in a sequential double infection, which would allow it to allocate more resources to growth. Such a lower cost could, for example, be due to the host's immune system focusing on the first cestode ('original antigenic sin'; Francis 1953; Fazekas de St. Groth and Webster 1966a, b). However, a parasite-driven rather than a host-driven explanation could also apply: immunosuppression by the first parasite. If the host's immune system is already suppressed by the first cestode at the time of the second infection, this could facilitate establishment and growth of the second cestode. This

idea finds support in a study by Scharsack *et al.* (2004), in which they investigated responses of immunologically active cells. They compared the responses of leukocytes from sham- and *S. solidus*-infected fish after the cells had been incubated *in vitro* in a medium, to which *S. solidus*-extract had been added. This setup can be considered analogous to a sequentially acquired double infection. While the leukocytes from sham-infected fish showed a stronger immune response when exposed to medium with *S. solidus* extract than when exposed to pure medium, the leukocytes from fish infected with *S. solidus* did not show such a difference in their response. This lack of a response by the leukocytes from the infected fish when exposed to *S. solidus* strongly suggests a suppression of the fish's immune system by the infection.

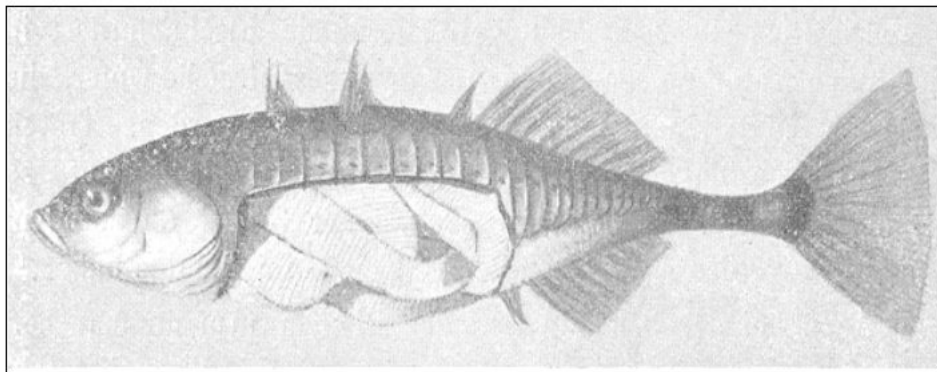
An important question to consider is whether within-host dynamics of double infections are of evolutionary relevance for this parasite or whether the fitness costs of being in a double infection are so vast that the dynamics within them are of only minor evolutionary importance compared to those of single infections. Although the focal cestode grew to a larger size when it was the second cestode in a sequential double infection than when it was in a simultaneous double infection, it was nevertheless almost 20% smaller than a focal cestode inhabiting the host alone (Fig. 3C). Since there is a strong positive correlation between the cestode's weight at the time of transmission to the final bird host and its fecundity in the bird (Wedekind *et al.* 1998), the smaller cestodes from double infections are expected to suffer a lower fecundity. However, this lower expected fecundity for cestodes in doubly exposed fish could be compensated for by a higher probability of becoming established in the fish. Knowing that, of the double exposures that turn out to be single infections, a focal cestode was found in 52.6% of them when the exposures were simultaneous and in 73% of them when the exposures were sequential (and the focal cestode thus the later cestode), we can estimate crude individual survival probabilities for focal cestodes from figure 1. Thus, while a cestode in a simultaneous double exposure can expect a survival probability of 36 - 51%, this probability increases to 47 - 62% when it is the secondly acquired cestode in sequential double exposures. Comparing these to a survival probability of 44% in singly exposed fish indeed suggest that a higher survival probability can partly or even fully compensate for the slower expected growth of cestodes in double exposures. Although very crude, these estimates emphasize that, for this parasite, the within-host dynamics of double infections and

their consequences for the cestode's fitness are indeed relevant and likely to play an important role in evolutionary terms.

In conclusion, we found that the genetic variation and the timing of double exposures of the cestode *S. solidus* affected the parasite's establishment and growth within its stickleback host. In male fish, exposure was most likely to result in an infection if the two cestodes were related, while female fish were more easily infected if the cestodes were unrelated. Irrespective of fish gender, the infection was more likely to contain both cestodes if they were related. In sequential double infections, the cestode in the last exposure was more likely to become established within the host. In addition, despite being one week younger, it grew to a larger size than the cestode from the first exposure. At this stage, we can only hypothesize about the mechanisms involved. However, our results emphasize that within-host dynamics and factors acting at this level can play an important and sometimes surprising role in determining a parasite's transmission success.

CHAPTER III

Effects of the cestode *Schistocephalus solidus* on innate and adaptive immune traits of the three-spined stickleback (*Gasterosteus aculeatus*)



Abstract

The cestode *Schistocephalus solidus* and its host, the three-spined stickleback (*Gasterosteus aculeatus*), represent an ideal model system to study coevolutionary host-parasite interactions. Previous *in vitro*-experiments suggest that *S. solidus* is capable of suppressing innate immune responses of the stickleback. However, little is known about the adaptive immune response. We repeatedly challenged the adaptive part of the immune system of *S. solidus*-exposed and sham-exposed sticklebacks *in vivo* by injecting *S. solidus* extract and DNP-BSA 5 and 7 weeks post-infection (p.i.). Parameters of innate and adaptive immunity were measured 9, 13 and 23 weeks p.i. *S. solidus* infection had a significant effect on the respiratory burst reaction of head kidney leukocytes 9 weeks p.i., but not 13 and 23 weeks p.i. No significant influence of *S. solidus* infection and antigenic challenge on lymphocyte proliferation, which was here used as a measure of adaptive immune activation, was detectable throughout the experiment. Interestingly, 9 weeks p.i., infected fish had a higher body condition than exposed but not infected sticklebacks, which might reflect the costs of clearing the infection. Later, the body condition of infected fish decreased, presumably due to the costs of parasite infestation. Our study confirms that, in response to *S. solidus* infections, innate immunity seems to be important. The role of adaptive immunity with respect to *S. solidus* infections is discussed.

Introduction

Parasite infections can have a large impact on their hosts, which might in turn lead to counter-adaptations in terms of resistance. Parasites might then evolve strategies to overcome the counter-adaptations, potentially resulting in an arms race (van Valen 1973). Apart from avoiding parasites (spatially or behaviourally) (Reimchen and Nosil 2001; Moore 2002) or building up mechanical barriers (e.g. a mucosal layer on the skin) (van Muiswinkel, 1995), higher parasite resistance can be achieved with an effective immune system. As a vertebrate, the stickleback is endowed with two lines of defence, which are, however, tightly connected (Dixon and Stet 2001; Luster 2002; Goldstein 2004): the innate and the adaptive (or acquired) arm of the immune system. While the innate arm of immunity represents a quickly available but rather unspecific protection against pathogens (but see Kurtz, 2005), the adaptive part takes some time to mount the highly specific antibody-based response (Janeway *et al.* 1999). *S. solidus* has been shown to induce a significant increase in immunologically active cells, i.e. leukocytes, in the peripheral blood of the three-spined stickleback (Barber *et al.* 2001a). Furthermore, sticklebacks were found to elicit a respiratory burst reaction following *S. solidus* infection (Kurtz *et al.* 2004; Scharsack *et al.* 2004). During the respiratory burst, which is considered to be an important mechanism of the fish innate immune system (Zapata *et al.* 1996; Whyte, *et al.* 1989; Nie and Hoole, 2000), granulocytes generate reactive oxygen species to kill pathogens. Scharsack *et al.* (2004) observed increasing proportions of granulocytes (i.e. cells of the innate immune system) and decreasing proportions of lymphocytes (i.e. cells of the adaptive immune system) in the head kidney (a major immune organ of fish (Zapata *et al.*, 1996)) and in the blood of *S. solidus*-infected sticklebacks up to 63 days p.i. Thereafter, these changes in the frequency of leukocyte subsets were again levelled out, although parasites were still growing. When investigating the role of individual diversity in major histocompatibility complex (MHC) class IIB genes, a key component of adaptive immunity, Kurtz *et al.* (2004) observed no significant effect on the infection rate of *S. solidus*. However, *S. solidus* exposed sticklebacks with an optimal MHC class IIB diversity showed a lower respiratory burst reaction and when infected, the parasites were found to have a reduced growth (Kurtz *et al.* 2004). Thus, involvement of adaptive immunity in the defence against *S. solidus* seems to be more complex.

Parasites, on the other hand, might counter-adapt to an effective host immune response by either evading or fighting the immune system. Earlier studies suggest that *S. solidus* has adopted various strategies to cope with the stickleback's immune system. Hammerschmidt and Kurtz (2005a) found that *S. solidus* changes its surface structure upon host switch from the first intermediate host, a copepod, to the second intermediate host, the stickleback. Moreover, variability in surface composition is related to parasite fitness in the second intermediate host. Both results indicate that *S. solidus* camouflages itself to remain undetected by components of the stickleback's immune system. A study by Scharsack *et al.* (2004) suggests that *S. solidus* also suppresses host immunity, a strategy that is well known to be adopted by many parasites including protozoans (Saeij *et al.* 2002; Boëte *et al.* 2003; Scharsack *et al.*, 2003), acanthocephalans (Rigaud and Moret 2003), nematodes (Hoerauf *et al.* 2005) and bacteria (Juris *et al.* 2002). With *in vitro*-experiments, Scharsack *et al.* (2004) showed that leukocytes from *S. solidus*-infected three-spined sticklebacks failed to show an elevated respiratory burst reaction upon immune challenge, whereas those from sham-exposed fish did. It thus seems as if the parasite is capable of suppressing innate reactions of the host's immune system. Further support comes from a study that investigated double infections of *S. solidus*. In sequential double infections, the secondly acquired cestode grew larger than its partner within the same host, despite being one week younger, whereas in simultaneous infections this difference did not occur (Jäger and Schjørring 2006). This could result from a competitive advantage a secondly acquired cestode might gain if the first cestode suppresses the host's immune system.

To further investigate the complexity of these interactions, we here addressed the question whether *S. solidus* is able to also suppress adaptive immune responses. We therefore challenged the immune system of exposed and sham-exposed fish *in vivo* by injecting a natural (*S. solidus* extract) and an artificial antigen (DNP-BSA) 5 and 7 weeks p.i. The combination of infection and subsequent immunisation with the two different antigens allows us to differentiate between a rather specific inefficiency of the stickleback's immune system towards *S. solidus* and a more general immunosuppression. In the first case, we would expect stronger immune reactions of infected fish to DNP-BSA than to *S. solidus* extract, while we would expect to find a similar weak reaction to either antigen in the case of a general suppression. We measured two parameters of the innate immune system (respiratory burst and

proportion of granulocytes in the head kidney) as well as one parameter of the adaptive immune system (proportion of proliferating lymphocytes in the head kidney) 9, 13 and 23 weeks p.i. In poikilothermic fish, the antibody response is temperature-dependent and it can take several weeks until the peak antibody response is present (Rijkers *et al.* 1980). Proliferation, i.e. clonal expansion, of lymphocytes in fish, as in other vertebrates, is a significant part in the generation of a specific adaptive immune response (van Muiswinkel 1995). Measurement of lymphocyte proliferation can therefore be used to describe the actual status of the adaptive arm of the immune system in fish (Nie *et al.* 1996; Hamers and Goerlich 1996; Scharsack *et al.* 2000). In sticklebacks, stimulation with mitogens *in vitro* led to an up-regulation of proliferating lymphocytes (Kurtz and Scharsack, unpublished data).

By sampling at three different points of time, we monitored the stickleback's immune responses over the course of infection. Since *S. solidus* is a naturally occurring parasite of the three-spined stickleback, we are here investigating potentially co-evolved immunological interactions under controlled laboratory conditions. So far, studies investigating immunological interactions between hosts and parasites have mainly used established laboratory systems, which often do not represent naturally co-occurring host-parasite systems.

Materials and Methods

Study system

The three-spined stickleback (*Gasterosteus aculeatus*) is the obligatory second intermediate host of the cestode *Schistocephalus solidus*. *S. solidus* has a complex life cycle involving three hosts. The first intermediate host is a cyclopoid copepod, from which the cestode is trophically transmitted to the second intermediate host, the three-spined stickleback. Growth takes place in the body cavities of both intermediate hosts, while reproduction occurs in the gut of the final host, a fish-eating bird.

The fish used in the present study were the laboratory-bred offspring of individuals that had been caught in the Große Plöner See in Plön (Northern Germany) in winter 2003/2004 (see Bakker (1986) for details of breeding sticklebacks in the laboratory). Similarly, the parasites were the laboratory-bred offspring of individuals that were removed from wild fish caught in the Neustädter Binnenwasser (Northern Germany) in autumn 2003 (for details of breeding the cestodes in the laboratory see Smyth

(1954), modified by Wedekind (1997)). At the time of infection the fish were about nine months old.

Experimental procedure

We used three different fish families. Each family was divided into two subgroups, one was exposed to one of the three different *S. solidus*-strains and one was sham-exposed. For the experimental infections, individual sticklebacks were transferred to 1 l aquaria and exposed to infected copepods (*Macrocyclops albidus*, for details of copepod infection see Christen *et al.* (2002)) harbouring in total three *S. solidus* larvae. Before being transferred to 200 l tanks three days later, fish were marked according to the treatment, i.e. the individuals of each treatment could be recognised by a specific spine code. This code consisted of two or three spines being cut per individual. The treatment-specific spine codes were altered among fish families. Within families, the experimental fish were randomly assigned to the different treatments. At the same time, weight (to the nearest mg) and body length (from the snout to the tip of the tail, to the nearest mm) for each individual was determined. The three fish families were kept separately, but within each family, individuals of all treatments were housed together. Housing conditions were kept constant at 18° C and a 16:8 h light:dark cycle. Fish were fed *ad libitum* three times a week with either frozen chironomid larvae or a mix of frozen daphnia and cyclops.

38 days after exposure to *S. solidus*, the first immunisation took place, it was repeated 13 days thereafter. Prior to injection, the fish were anaesthetised with MS 222 (0.2 g/l). They were then injected intraperitoneally with either 10 µl of *S. solidus* extract (see below), Dinitrophenyl-Bovine Serum Albumin (DNP-BSA; Calbiochem, USA) or Phosphate Buffered Saline (PBS) as a control. All three reagents were mixed with Freund's Complete Adjuvant (Sigma-Aldrich, USA) in a proportion of 1:1. Fish had a body weight of approximately 1 g, thus all reagents were applied at a final concentration of approximately 5 µg protein/g body weight. The *S. solidus* extract was obtained by homogenisation of whole cestodes retrieved from sticklebacks (for details see Scharsack *et al.* (2004)).

This resulted in six different treatments: (I) exposed to *S. solidus* and immunised with DNP-BSA, (II) non-exposed to *S. solidus* and immunised with DNP-BSA, (III) exposed to *S. solidus* and immunised with *S. solidus* extract, (IV) non-exposed to *S.*

solidus and immunised with *S. solidus* extract (V) exposed to *S. solidus* and immunised with PBS and (VI) non-exposed to *S. solidus* and immunised with PBS.

Samples were taken at three different points of time on two consecutive days (65 and 66 days, 93 and 94 days, 162 and 163 days after exposure to the parasite). Each fish's weight, body length and sex was determined. The fish were killed by a cerebrosplinal cut and when infected, the parasite(s) were removed from the body cavity and weighed individually (to the nearest mg). Thereafter, head kidneys were taken from each individual fish for immunological analysis.

For each treatment group, a mean initial and an individual final body condition factor was calculated as $100 \times \frac{W}{L^b}$ where W is the fish weight in g, L the fish length in cm and b the regression coefficient calculated from the logarithm-transformed values of initial length and initial weight (Frischknecht 1993). For the final body condition, the regression coefficient was calculated for each sampling date separately, i.e., only the values of the fish being dissected at the respective time were computed.

Immunoassays

To determine the relative number of granulocytes, leukocyte suspensions were prepared from the individual head kidneys. The leukocytes were processed and analysed using a Becton Dickinson FACSCalibur flow cytometer and the *Cellquest Pro* 4.02 software as described in Hammerschmidt and Kurtz (2005b). For the adjustment of cell numbers for the subsequent respiratory burst assay, absolute cell counts were determined with the standard cell dilution assay (Pechhold *et al.* 1994, modified by Scharsack *et al.* 2004). We analysed the respiratory burst (relative luminescence units (RLU)) associated with phagocytosis of zymosan particles *in vitro* in a lucigenin-enhanced chemiluminescence assay (modified after Scott and Klesius 1981 as described in Kurtz *et al.* 2004). The cell density of each fish sample was adjusted to 1.25×10^6 cells ml^{-1} , which corresponds to 2×10^5 cells per well of a 96-well-plate. Luminescence was measured for three hours at 20°C.

As a measure of adaptive immune function, we determined the relative number of proliferating head kidney lymphocytes. Cells can be associated with different phases of the cell cycle (G₀₁-, S- or G_{2M}-phase) according to the DNA content since it is increased in proliferating cells. To isolate the lymphocytes, 100 μl of the above leukocyte suspension was fixed with 900 μl of Ethanol (99.8%) and stored at 4°C.

Before further processing, cells were centrifuged (10 min, 500 x g) and Ethanol was removed. After degradation of RNA with 250 µl of RNase (500 µg/ml PBS, 10 min) we added 150 µl of propidium iodide (20 µg/ml PBS, Sigma Aldrich, Saint Louis, USA) to quantify the amount of DNA present in each cell. Each fish sample was measured for three minutes or up to 30,000 events on a Becton Dickinson FACSCalibur flow cytometer using the *Cellquest Pro* 4.02 software. Cellular debris (low scatter characteristics) and aggregated cells (high scatter characteristics) were excluded from further evaluation. Doublet cells were differentiated from single cells as described by Wersto *et al.* (2001). Lymphocytes were identified according to their characteristic FSC/SSC profiles and could be associated with the G₀₁-, S- and G_{2M}-phase according to their red fluorescence intensity (propidium iodide labelling of DNA content). Lymphocytes in S- and G_{2M}-phase were considered to be proliferating.

Statistical Analysis

Data from the two consecutive days of the single sampling dates were pooled. We observed large variation in responses between the three sampling dates, which were, however, not due the experimental treatments since all fish were affected likewise. Therefore, the three points of time were analysed separately.

To rule out potential effects of fish family, fish gender and initial body condition on the parameters of interest, we corrected for these factors by using the residuals of an analysis of variance (ANOVA) incorporating fish family, fish gender and initial body condition for all further statistical analyses. To test for significant effects on the immune parameters as well as on the body condition of the fish, ANOVAs with antigen injection and infection status as two independent variables were conducted. Further ANOVAs were calculated to test whether the total parasite weight was influenced by the immune reactions measured. All interactions were initially included in the models and insignificant ones subsequently removed (at the 10% significance level) by performing backward elimination. When significant, a post-hoc test (after Tukey) to obtain the respective significance levels was applied. To meet the assumptions of normality, data were transformed when necessary. For all statistical analyses, the software JMP 5.0.1.2 for Macintosh (SAS Institute Inc. 2003) was used.

Results

Of the 197 fish that underwent the experimental procedure, 24 died during the experiment. Since they were randomly distributed with regard to treatment, they were excluded and a total of 173 fish was considered in the final analysis. Out of those, 90 (52.0%) were sham-exposed, 27 (15.6%) were infected with at least one cestode and 56 (32.4%) were exposed but not infected. Variable sample sizes in the following analyses are due to the fact that for some individuals the number of head kidney leukocytes was not sufficient for all immunological assays.

Surprisingly, neither *S. solidus* extract nor DNP-BSA showed any significant influence on the innate or adaptive immune traits measured (Table 1A). There was also no significant effect of the injected antigens on the body condition of the fish (Table 1A). Furthermore, a fish's infection status did not affect its response to the antigens since the interaction was not significant for any of the variables.

There was, however, a significant effect of infection status on the respiratory burst reaction 9 weeks p.i. with fish harbouring at least one parasite showing a stronger response (Table 1B, Fig. 1). No such effect was found 13 and 23 weeks p.i. (Table 1B, Fig. 1). However, total parasite weight was not significantly affected by the respiratory burst reaction 9 weeks p.i. (ANOVA, $N = 8$, $df = 1$, $F = 0.02$, $P = 0.885$).

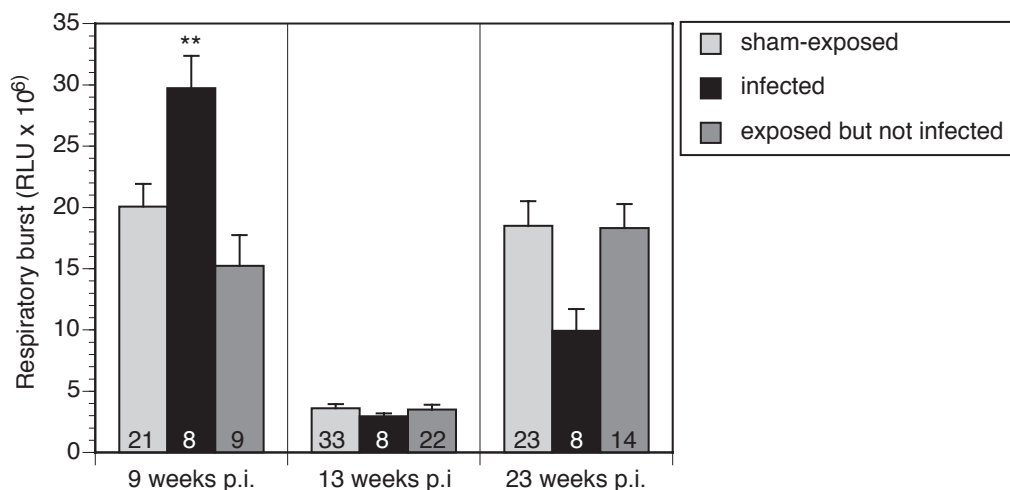


Figure 1: Respiratory burst activity (relative luminescence units (RLU)) of isolated head kidney leukocytes from sticklebacks exposed and sham-exposed to the cestode *S. solidus* at different times p.i. Sample sizes are given at the bottom of the bars. Error bars indicate one standard error. Significant differences are indicated with asterisks (**: $P < 0.01$).

Neither the proportion of granulocytes nor the proportion of proliferating head kidney lymphocytes was significantly influenced by infection status at any of the three sampling dates (Table 1B, Fig. 2 and Fig. 3 respectively). At the first sampling, the proportion of proliferating head kidney lymphocytes was only examined on the second experimental day, which resulted in a lower sample size; the treatments were, however, all represented.

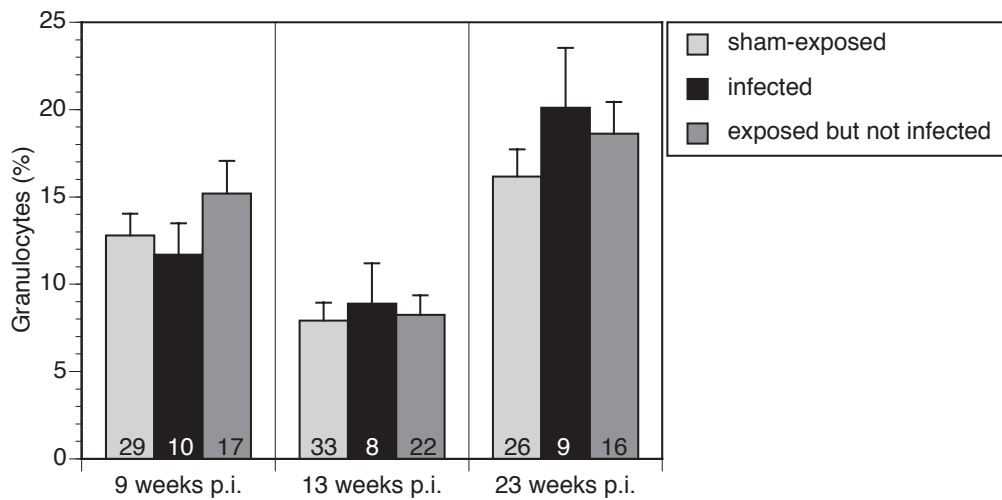


Figure 2: Proportion of granulocytes in isolated head kidney leukocytes from sticklebacks exposed and sham-exposed to the cestode *S. solidus* at different times p.i. Sample sizes are given at the bottom of the bars. Error bars indicate one standard error.

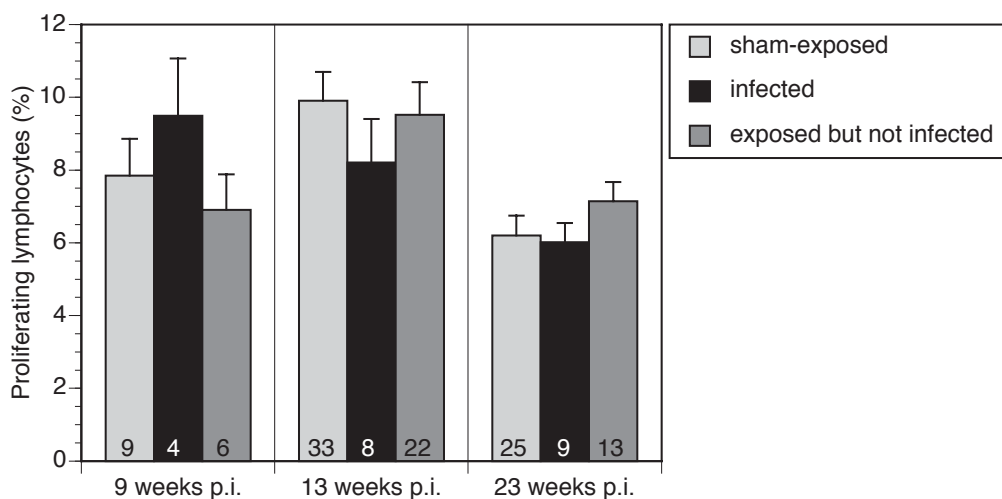


Figure 3: *Proportion of proliferating lymphocytes in isolated head kidney leukocytes from sticklebacks exposed and sham-exposed to the cestode *S. solidus* at different times p.i. Sample sizes are given at the bottom of the bars. Error bars indicate one standard error.*

Concerning the effect of infection status on the body condition index of the fish, there was a trend 9 weeks p.i. for fish harbouring at least one cestode to have a higher body condition than those that were exposed but not infected (Table 1B, Fig. 4). 13 weeks p.i., this finding was reversed and the infected fish were found to have a significantly lower body condition than the exposed but not infected fish (Table 1B, Fig. 4). This pattern remained unchanged 23 weeks p.i., although only as a trend (Table 1B, Fig. 4).

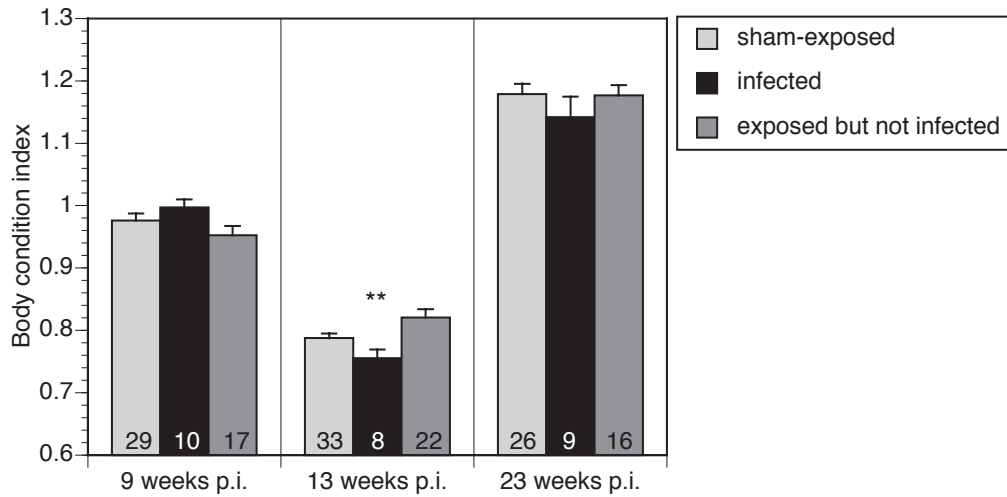


Figure 4: *Body condition index of sticklebacks exposed and sham-exposed to the cestode *S. solidus*. Measurements were taken at different times p.i. Sample sizes are given at the bottom of the bars. Error bars indicate one standard error. Significant differences are indicated with asterisks (**: $P < 0.01$).*

Dependent variable	df	9 weeks p.i.			13 weeks p.i.			23 weeks p.i.		
		N	F	P	N	F	P	N	F	P
A Respiratory burst	2	38	0.82	0.449	63	0.96	0.388	45	2.32	0.112
% granulocytes	2	56	0.77	0.469	63	0.19	0.831	51	0.40	0.672
% proliferating lymphocytes	2	19	0.59	0.570	63	0.75	0.476	47	0.61	0.550
Body condition	2	56	1.30	0.282	63	1.70	0.191	51	0.31	0.739
B Respiratory burst	2	38	7.21	0.003	63	0.04	0.957	45	1.29	0.288
% granulocytes	2	56	0.08	0.927	63	0.81	0.450	51	1.39	0.260
% proliferating lymphocytes	2	19	1.31	0.300	63	0.02	0.977	47	1.33	0.277
Body condition	2	56	2.58	0.086	63	6.60	0.003	51	2.95	0.062

Table 1: Effect of (A) antigen injections (*S. solidus* extract, DNP-BSA and PBS) and (B) infection status on immune traits and body condition of the three-spined stickleback at different times after exposure to *S. solidus*. ANOVAs with antigen injection and infection status as two independent variables were conducted to test for the effects. Since not significant, the interaction term was removed from all models. For all tests, residuals of an ANOVA incorporating fish family, fish gender and initial body condition were used. Injections took place 5 and 7 weeks p.i. Respiratory burst and % granulocytes represent parameters of the innate immune system of the stickleback, while % proliferating lymphocytes reflects a parameter of the adaptive immune system.

Discussion

Infection with the cestode *S. solidus* was found to elicit an innate immune response in the three-spined stickleback 9 weeks p.i.: fish infected with at least one cestode showed a significantly higher respiratory burst reaction than sham-exposed fish and fish that were exposed but not infected (Fig. 1). *S. solidus* has been shown to induce a respiratory burst reaction before and, as in our study, this was the case around 9 weeks p.i. (Kurtz *et al.* 2004; Scharsack *et al.* 2004). Together these studies emphasise the importance of innate immune mechanisms in the defence against *S. solidus*. Such an elevated respiratory burst response could be due to an increase in the proportion of granulocytes as indicated by an earlier study (Scharsack *et al.* 2004). In our study, however, infected fish did not differ significantly from sham-exposed fish in their proportions of granulocytes (Fig. 2), which indicates an elevated respiratory burst capacity of single cells rather than increased granulocyte numbers. Kalbe and Kurtz (2006) also suggested such an up-regulation of single cell capacity for sticklebacks infected with the eye fluke *Diplostomum pseudospathaceum*. Kurtz *et al.* (2004) found indications for both, an increase in granulocyte numbers as well as an up-regulation of single cell capacity for *S. solidus*-infected sticklebacks.

Innate immune responses, like the respiratory burst reaction, are fast and generally precede adaptive immune responses. Since the innate and the adaptive part of the immune system are mutually regulated (Janeway *et al.* 1999; Luster 2002), we might expect a down-regulation of the innate immune system, i.e. the respiratory burst reaction, followed by an up-regulation of the adaptive immune system if the infection has not been cleared. After the peak 9 weeks p.i., we did observe a decline of the respiratory burst back to the level of the control group, the non-exposed fish (Fig 1); we did, however, not find a follow-up increase in the proportion of proliferating lymphocytes (Fig. 3). Although it has to be treated with caution since we only measured one parameter of adaptive immunity, this might indicate that the adaptive part of the stickleback's immune system could play a smaller role with respect to *S. solidus* infections. Scharsack *et al.* (2004) also observed a reversion of the proportion of granulocytes after an initial increase but no follow-up increase of the proportion of lymphocytes. Another study suggested that individual diversity of MHC class IIB alleles, which plays an important role in the adaptive antibody-based immune response, might not affect the establishment of *S. solidus*, but is then relevant during

later stages of the infection (Kurtz *et al.* 2004). However, individual MHC class IIB diversity has also been shown to be related to the innate respiratory burst reaction since individuals carrying an optimal diversity showed a lower respiratory burst reaction (Kurtz *et al.* 2004). Further investigations are needed to understand the role of adaptive immunity in *S. solidus* infections. The next step would clearly be the development of antibodies directed against stickleback immunoglobulin to enable detection of potential specific antibodies during the course of an infection.

Surprisingly, we did not find any indication that the injection of a natural (*S. solidus* extract) or an artificial (DNP-BSA) antigen stimulated any of the immune parameters measured nor had it any influence on the fish's body condition (Table 1A). Since there was also no response to the injections in the sham-exposed fish, the lack of a response in *S. solidus*-exposed fish cannot be due to the hypothesised suppression by *S. solidus*. The *S. solidus* extract used in this study has been shown to induce an immune response *in vitro* in the three-spined stickleback in an earlier study (Scharsack *et al.* 2004) and the dose we applied (5 µg/g fish body weight) is within the range of doses used in previous immunisation studies with fish: doses ranged from about 1 µg/g body weight for whole worm homogenate of the cestode *Ligula intestinalis* applied to roach (*Rutilus rutilus*, Williams and Hoole 1992) to about 40 µg/g body weight for ciliate lysate applied to turbot (*Scophthalmus maximus*, Iglesias *et al.* 2003). Similarly, a comparable antigen, Dinitrophenyl-Human Serum Albumin (DNP-HSA), was found to induce an adaptive immune response in turbot (Kofod *et al.* 1994) and salmon (*Salmon salar*, Nielsen and Buchmann 1997). In summary, the immunisation protocol used in the present study is in accordance with previous investigations and it is not clear, why no detectable immune response was induced in the present study.

Infection status affected the body condition of the fish and, more interestingly, this influence changed during the course of infection (Fig. 4). At the first sampling, 9 weeks p.i., there was a trend for the infected fish to have a higher body condition than the ones that were exposed but were not infected. This might reflect the costs of clearing the infection since immune reactions have been shown to be costly in terms of energy or immunopathology (Lochmiller and Deerenberg 2000; Kurtz *et al.* 2006). At this stage, the costs of infection might be negligible because the cestodes are still rather small. Moreover, the fish might still be able to compensate for the loss of resources by increasing their food intake, especially under laboratory conditions with

food *ad libitum*. Four weeks later, this finding was reversed and the infected fish had a significantly lower body condition than the exposed but not infected fish. This pattern was also found at the last sampling date. It is very likely that this is due to the impact the infection has on the host, i.e., the cestode acquiring resources, the activation of the immune system or both.

In conclusion, our study supports the hypothesis that stickleback innate immune mechanisms are important with respect to *S. solidus* infections. They may, however, carry costs for the host. The role of adaptive immunity in the defence against *S. solidus* is still not clear. Similarly, whether or not *S. solidus* is capable of immunosuppressing adaptive immune responses deserves further study.

CHAPTER IV

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



Abstract

Genes of the major histocompatibility complex (MHC) play a major role in pathogen resistance. Sexual selection combined with an ever-changing pathogen community has been hypothesized to maintain the striking polymorphism of those genes. In the three-spined stickleback (*Gasterosteus aculeatus*) there is strong evidence that an intermediate rather than a maximal individual MHC class IIB allele diversity is optimal with regard to parasite resistance. Here, we related individual MHC diversity to fitness traits likely to be affected by parasitisation, namely the number of egg clutches spawned by a female, male nest quality and the intensity of male breeding colouration. For the first time, MHC class I was investigated in three-spined sticklebacks as well. The sticklebacks under investigation were subjected to either high or low food conditions. The number of egg clutches as well as male nest quality was significantly higher under good food conditions. Whereas for male nest quality an optimum concerning MHC class IIB diversity was found, male breeding colouration was most intense at a maximal MHC class I diversity. Two MHC class I alleles were not only associated with a higher intensity of red colouration but were also more likely to be carried by individuals with a higher allele diversity, possibly explaining the positive correlation. It seems likely that these alleles confer resistance to a specific and presumably common intracellular pathogen.

Introduction

The major histocompatibility complex (MHC) has received much attention with respect to important questions of evolutionary ecology like parasite-mediated selection (e.g. Doherty and Zinkernagel 1975; Hedrick and Kim 1999; Wegner *et al.* 2003a, b) and sexual selection (e.g. Yamazaki *et al.* 1976; Potts *et al.* 1991; Reusch *et al.* 2001; Bonneaud *et al.* 2006; reviewed in Penn and Potts 1999; Milinski 2006). The MHC is central to the specific antibody-based responses of the adaptive (or acquired) immune system of vertebrates and contains the most polymorphic gene loci known in vertebrates (e.g. Janeway *et al.* 1999). MHC molecules are involved in pathogen recognition by presenting pathogen-derived peptides to T-cells that subsequently initiate an immune response. There are two classes of MHC molecules, class I and II. While MHC class I molecules bind peptides derived from intracellular pathogens, e.g. viruses, MHC class II molecules recognise peptides from extracellular pathogens. The striking polymorphism within those genes might be attributed to the balancing selection of an ever-changing pathogen community (Takahata and Nei 1990; Slade and McCallum 1992). In order to be able to fight a broad range of pathogens, one would expect selection to favour individuals with a high individual MHC diversity, i.e. high individual MHC heterozygosity. However, an increased number of MHC variants may not only bind more foreign antigens but also a greater variety of self-derived peptides, which may lead to an elevated risk to initiate an autoimmune response. T-cell clones that recognise self-peptides bound to MHC molecules are eliminated from the initially highly variable repertoire, thereby reducing the T-cell clone repertoire that is finally available for parasite recognition (Goldrath and Bevan 1999). A balance between the two opposing selective forces of broader pathogen resistance on one hand and increased T-cell clone elimination on the other hand might lead to an optimal rather than a maximal individual MHC diversity (Nowak *et al.* 1992; De Boer and Perelson 1993; Milinski 2006; but see Borghans *et al.* 2003).

The three-spined stickleback (*Gasterosteus aculeatus*) has been in focus of many MHC related investigations (e.g. Reusch *et al.* 2001; Aeschlimann *et al.* 2003, Wegner *et al.* 2003a,b; Kurtz *et al.* 2004; Kurtz *et al.* 2006; Wegner *et al.* 2006). There are probably six loci of MHC class IIB and twelve loci of MHC class I (Sato *et al.* 1998). Unlike in other vertebrates, the MHC class I and class II regions are not linked in teleost fish (Sato *et al.* 2000). In the stickleback, evidence for the existence

of an optimal rather than a maximal (i.e. 12 alleles) individual MHC class IIB diversity is mounting, whereby the optimal number of variants seems to be around six alleles. The mean individual diversity in a natural population was found to be 5.8 alleles (Reusch *et al.* 2001). Correlating individual MHC diversity of wild caught sticklebacks with their parasite load revealed a minimal parasite burden in fish with 5.2 MHC class IIB alleles (Wegner *et al.* 2003b). This correlative observation was later confirmed in two experimental studies, in which individuals with an intermediate number of MHC class IIB alleles (i.e. 5-6 alleles) suffered least from parasite infection and were able to reduce parasite growth (Wegner *et al.* 2003a; Kurtz *et al.* 2004). As yet, MHC related studies in the stickleback were restricted to MHC class IIB, thereby missing out on the whole range of intracellular pathogens.

As in many other vertebrate taxa (e.g. reviewed in Penn and Potts 1999 and Penn 2002; Bonneaud *et al.* 2006) mate choice in the three-spined stickleback has been shown to be related to MHC (Reusch *et al.* 2001; Aeschlimann *et al.* 2003; Milinski 2003). In contrast to many other studies, which found disassortative mating preferences (e.g. Yamazaki *et al.* 1976; Wedekind *et al.* 1995; Olsson *et al.* 2003; Richardson *et al.* 2005), stickleback females did not prefer MHC dissimilar mating partners to MHC similar ones (Reusch *et al.* 2001). Rather they chose partners such that, in self-reference to the number of her own alleles, the mean MHC IIB allele diversity of the offspring would be 5.2 (Aeschlimann *et al.* 2003). So far, MHC based female mating preference has only been related to odour signals. However, stickleback mate choice possibly depends on numerous male secondary sexual traits like breeding colouration (Milinski and Bakker 1990; Bakker and Milinski 1991; Milinski and Bakker 1992), nest quality (Barber *et al.* 2001b) and zigzag dance (Rowland 1995). Females are expected to assess their mates by traits honestly reflecting their condition (Zahavi 1975; Hamilton and Zuk 1982). Indeed, the intensity of the male red breeding colouration has been shown to reveal condition and parasitisation, and females chose, as expected, the redder unparasitised male (Milinski and Bakker 1990). Despite this close connection between the immune status of a male and its breeding colouration, male redness has not been related to MHC based pathogen resistance yet. Similarly, other fitness related traits like the male secondary sexual traits mentioned above or female traits such as the number of egg clutches might reflect an individual's immune status.

MHC based pathogen resistance might be more important under poor food conditions since trade-offs are often weaker or sometimes not even visible at all under good environmental conditions (Stearns 1992). If food is limited, a potentially costly immune response can possibly not be compensated by a higher food intake.

In the present study, we related individual diversity of MHC class IIB and MHC class I to relevant fitness traits of the three-spined stickleback, namely the number of egg clutches spawned by a female, male nest quality and the intensity of male breeding colouration. This is the first study to investigate MHC class I in the three-spined stickleback. Investigating sticklebacks under high and low food conditions might enable us to evaluate the relative importance of MHC diversity for individual fitness.

Materials and Methods

Study animals

Three-spined sticklebacks were caught in the Große Plöner See (Northern Germany) in December 2003. In the laboratory, they passed through spring temperature and light conditions before they were kept under summer conditions (18°C, 16:8 h light:dark cycle) for the rest of the time. Each fish's length (to the nearest mm) and weight (to the nearest mg) was measured about six weeks after catching (when the fish were transferred from spring to summer conditions). A body condition factor was calculated as $100 \times \frac{W}{L^b}$ where W is the fish weight in g, L the fish length in cm and b the regression coefficient calculated from the logarithm-transformed values of length and weight (Frischknecht, 1993). In the following, this body condition index is referred to as initial body condition. Under summer conditions, fish were fed *ad libitum* with frozen chironomid larvae and additional live food every day (well-fed group) or only frozen chironomid larvae every second day (poorly fed group). Individual fish were randomly assigned to the two food groups.

MHC class I and class II genotyping

Genomic DNA was extracted from a single dorsal spine with a DNA extraction kit (Invitex, Germany) from each individual fish. The MHC class IIB exon 2 (*I* domain) diversity was determined by using capillary electrophoresis (CE) single strand conformation polymorphism (SSCP) as described in Binz *et al.* (2001). Recently, CE-

SSCP genotyping has been extended to determine individual MHC class I exon 2 (*I* domain) diversity in the three-spined stickleback (Schaschl and Wegner, in press). In brief, PCR was carried out by using the MHC class I forward primer GAAC3 (Sato *et al.* 1998) and the following three class I sequence-specific reverse primers (primers were differently labelled on the 5' end with either FAM, HEX or TET): GaIRo01 (5'-AGT TTG GTT GAA GCG TTG TTT TGC-3'), GaIRo02 (5'-AGT TTG GTT GAA GCG TYG TTT CAC-3'), and GaIRo03 (5'-TTG GTT GAA GCG TYG TTT TAG-3'). The forward primer GAAC3 was used in combination with the dye labelled primer GaIRo01 in a first PCR reaction and with a GaIRo02/GaIRo03 primer mix in a second one. The thermal cycling profile for both PCRs was: initial heating at 95°C for 15 min (hot-start polymerase activation), followed by 30 cycles of denaturation at 94°C for 35 s, annealing at 56°C for 35 s, extension at 72°C for 1 min and ending with a 10 min extension step at 72°C. Fluorescent-labelled PCR samples were prepared for electrophoresis by combining 1 µl PCR product with 9 µl loading mix (8.35 µl Hi-DI formamide, 0.35 µl Genescan ROX 350 standard (Applied Biosystems) and 0.3 µl 0.3N NaOH). The mixture was heated for 5 min at 92°C to separate the complementary DNA strands, chilled on ice and analysed by capillary electrophoresis on an ABI PRISM® 3100 automated DNA Sequencer (Applied Biosystems). The separation of the class I allelic variants was achieved under native capillary electrophoresis by a run temperature of 22°C. The retention times of the MHC allelic variants were identified relative to the ROX 350 standard. The GeneScan and Genotyper software packages (Applied Biosystems) were used to process the obtained SSCP data.

Number of egg clutches, nest quality and male breeding colouration

During 25 days, starting approximately four weeks after fish had been moved to summer conditions, each female's gravidity was assessed every day except day 15 and 20. A female was assumed to have spawned when assessed as being very ripe on one day but not on the following. When ripe, female sticklebacks eventually spawn their eggs even if no male is available.

Assessment of male nest quality took place on day 25, the last day of female assessment. Nests were classified according to five categories: 0 – no nest; 0.5 – nest messy, no visible entrance; 1 – nest messy, entrance vaguely visible; 1.5 – nest neatly

glued, but entrance only vaguely visible; 2 - nest neatly glued and entrance clearly visible. The rationale behind this classification was to assess how well the nest was maintained, i.e. if it was glued regularly, also by the male creeping through. An only vaguely visible entrance represents an opening that is not regularly used and glued. All nests were assessed by the same person.

The red breeding colouration was analysed about one week after the assessing period. For that, males were photographed ventrally after having been stimulated with a ripe female for 15 min the day before. The males were gently fixed with a sponge in a water-filled plastic box, which had a window of filter glass (high resolution skylight filter, Hama, Germany) to ensure that the whole colour spectrum was transmitted. The pictures were taken within a dark box using a digital camera (Olympus E20-p) with a 36 mm macro-objective. Closing time of the lens was 1/60 sec with aperture 7. For illumination, we used 4 cold lights (KL 1500 LCD, Leica) with 3300 K colour temperature according to daylight illumination. Intensity analysis of the red colouration was performed with IP Lab 3.6.2 for Mac OS 9.2.2 (Scanalytics, Inc.). On each picture, we selected an area of the throat covering the majority of the red parts (ROI (region of interest), see Fig. 1 for details). The mean red intensity was determined using the R/RGB model (8-bit red-green-blue colour model, Frischknecht 1993). All pictures were measured twice in random order, on two consecutive days. The marking of the area and the resulting red values were highly repeatable: 95.9% for the marked area as well as 99.98% for the mean colouration.



Figure 1: *Digital photograph of a male throat from below. Mean intensity of the red breeding colouration was analysed for the marked region (ROI (region of interest)) as described in the text.*

Parasites

Only males were dissected and screened for parasites. Since not all dissections could be done on the same day, only parasite species for which we were sure that they could not be eliminated during that time or during the assessment period were taken into account: the three trematode species *Diplostomum sp.*, *Cyathocotyle prussica* and *Echinochasmus sp.* as well as the nematode *Contracaecum sp.* Individuals of the respective parasite species were counted and an overall parasite load was calculated for each fish using the following approach (Rauch *et al.* 2006): for each parasite species, a relative number of individuals per fish was determined (number of individuals per fish divided by the maximum number of individuals of that respective species found on a single fish). The relative numbers of the four parasite species were summed up and averaged over the four species.

Statistical analysis

To analyse the influence of food group and individual diversity of MHC class I and MHC class IIB on the number of egg clutches, an analysis of variance (ANOVA) was conducted. Parasite load could not be considered since there was no data available for the females. Their initial body condition was not taken into account either because it could not be excluded that some individuals had already developed eggs at the time of measurement.

Males were tested for significant effects on nest quality with a generalized linear model (GLM) incorporating food group, number of MHC class I alleles, number of MHC class IIB alleles and parasite load as independent variables and initial body condition as a covariate. The same factors were considered with respect to male breeding colouration performing an analysis of covariance (ANCOVA).

All interactions were initially included in the models and non-significant ones subsequently removed (at the 10% significance level) by performing backward elimination. Significant single factors that are also part of significant interactions are considered when a post-hoc test (t-test or Chi-square test) verified its sole significance.

Chi-square tests - or two-tailed Fisher's Exact Tests when sample sizes were too small - were used to test for significant differences in allele frequencies when comparing the reddest and dullest quarter of the test fish. To reveal whether the presence of the

alleles identified by the above analyses increases with increasing individual allele diversity regression analyses were conducted. To account for the fact that only few individuals were available carrying allele numbers at the marginal ends of the allele range, the regression analyses were weighed by sample size. Finally, Oneway-ANOVAs were performed to test for significant effects of the presence of those alleles on male redness. A post-hoc test (Student's t-test) was applied to obtain the respective significance level.

All statistical analyses apart from the GLM were performed using the software JMP 5.0.1.2 for Macintosh (SAS Institute Inc. 2003). The GLM was conducted in R statistical package version 2.1.1.

Results and Discussion

Number of egg clutches

Females under good food conditions laid significantly more egg clutches than poorly fed females (ANOVA, $N = 75$, $df = 1$, $F = 43.04$, $P < 0.0001$, Fig. 2). Wootton (1994) and Fletcher and Wootton (1995) concluded that the number of clutches spawned per breeding season is dependent on the current food supply, whereas clutch and egg size are a function of female size at the start of the breeding season, thereby being only little affected by food availability. This would mean that egg size and the number of eggs per clutch are fixed before the start of the breeding season on the basis of current condition and food shortages occurring afterwards are then traded off by reducing the number of spawnings. However, current food availability seems to have a higher impact on female fitness since reducing the number of clutches also reduced the total number of eggs spawned within a breeding season (Wootton 1994). The poor food conditions applied in this study are not insufficient and probably still better than the food conditions encountered in nature; nevertheless, we found a big difference between the two food groups. This clearly emphasizes the importance of diet on individual performance and proposes to cautiously think about the feeding regime when conducting laboratory experiments (see also Barber *et al.* 2000; Candolin and Voigt 2001).

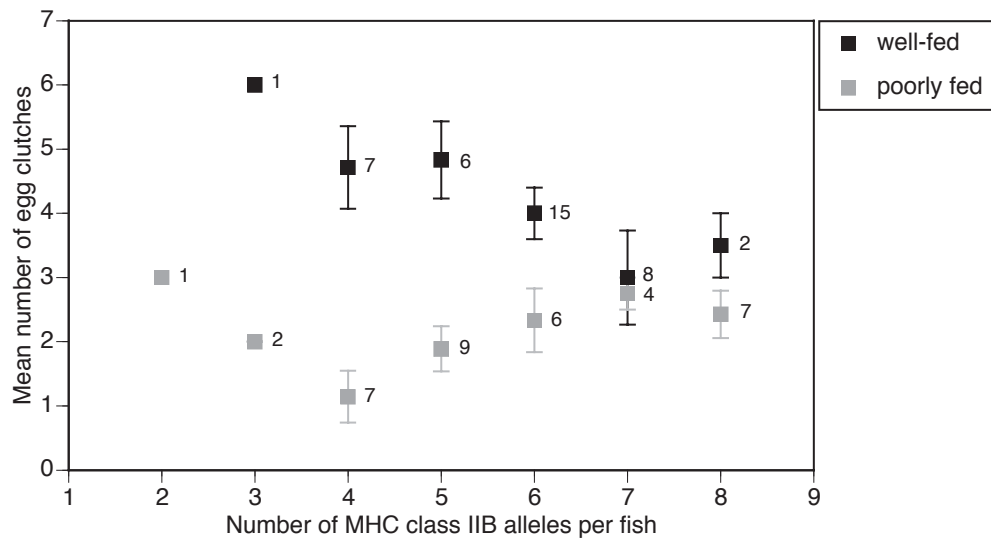


Figure 2: Mean number of egg clutches laid by female sticklebacks during the 25-day-assessment time in relation to individual MHC class IIB allele diversity. Sample sizes are given besides each data point. Error bars indicate one standard error.

It seems that it is not current food availability alone that determines the number of egg clutches a female spawns. We found a significant interaction between food level and individual MHC class IIB diversity on the number of egg clutches (ANOVA, $N = 75$, $df = 1$, $F = 11.10$, $P = 0.001$, Fig. 2). It might be that the importance of parasite resistance provided by adaptive immunity is higher when current energy availability is low. Due to the high immunopathology caused by, for instance, oxidative stress, innate immune mechanisms are thought to be very costly (von Schantz *et al.* 1999; Graham *et al.* 2005). When MHC-based adaptive immunity is efficient, an individual could be able to down-regulate the costly innate immune response (Kurtz *et al.* 2004; Kurtz *et al.* 2006). While a non-optimal individual encountering good food conditions might still be able to compensate the costs of a higher innate immune response, it might have to re-allocate energy to the costly innate immune response under poor food conditions. Since the number of egg clutches is highly affected by current energy income (Wootton 1994) such re-allocation might result in a reduced number of clutches in females with a non-optimal number of MHC IIB alleles. This might explain the increase in clutch number at an intermediate number of MHC class IIB alleles (5 to 7 alleles) in the poorly fed group (Fig.2). Considering the above hypothesis, one would not expect a decrease in clutch number at an intermediate allele number in the well-fed group (Fig. 2). However, the 25 days during which female

clutches were surveyed do not represent a whole stickleback breeding season. It might be that individuals with non-optimal allele diversity, although being able to compensate costly innate immune responses, invest more in early reproduction, whereas individuals with an optimal number invest during the whole breeding season.

Nest quality

As in females, food condition significantly affected male performance. Well-fed males maintained a nest of significantly higher quality than poorly fed males (GLM, $N = 72$, $df = 1$, $F = 5.88$, $P = 0.019$). Maintaining a high quality nest involves regular glueing, which might require a higher glue production. The glue is produced in the males' kidneys and they were significantly bigger in males that owned a nest of high quality (Barber *et al.* 2001b) as well as in males that were kept under good food conditions (Wootton 1994).

There was a significant effect of individual MHC class IIB diversity on nest quality (GLM, $N = 72$, $df = 1$, $F = 5.82$, $P = 0.019$). This effect is due to the fact that most of the individuals maintaining a high quality nest were carrying an intermediate number of MHC class IIB alleles, whereas most individuals with a low or high allelic diversity had nests of lower quality (Fig. 3). The optimum, i.e. the number of alleles at which the highest proportion of individuals owned a nest of the highest quality, was 6 alleles (Fig. 3). This is well in accordance with previous correlative and experimental studies that also found an optimal individual diversity of around six MHC class IIB alleles (Reusch *et al.* 2001, Aeschlimann *et al.* 2003; Milinski 2003; Wegner *et al.* 2003a, b; Kurtz *et al.* 2004).

Regarding MHC class I, there was a significant interaction between food level and allele diversity on nest quality (GLM, $N = 72$, $df = 1$, $F = 4.99$, $P = 0.029$). However, the range of individual allelic diversity was much smaller in the poorly fed than in the well-fed group (a range of 6 – 10 different MHC class I alleles in the poorly fed versus a range of 4 – 12 different alleles in the well-fed group). Thus, this result should be treated with caution. Food level also interacted significantly with parasite load on nest quality (GLM, $N = 72$, $df = 1$, $F = 17.02$, $P < 0.001$) and there was also a significant interaction with initial body condition and parasite load on nest quality (GLM, $N = 72$, $df = 1$, $F = 6.69$, $P = 0.012$). It thus appears that factors affecting nest

quality are more complex. A fish's condition (initial and current), MHC class I diversity as well as its parasite load seem to have an influence.

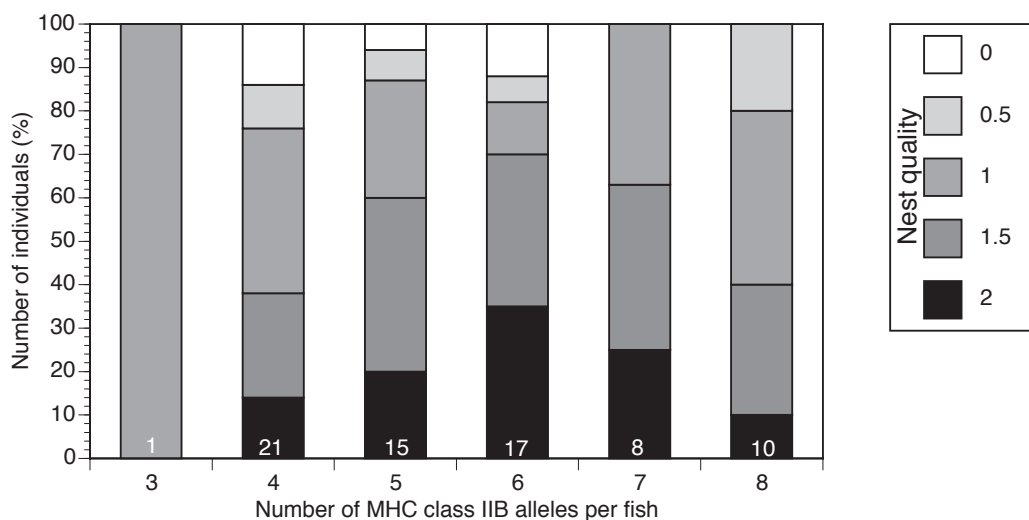


Figure 3: Proportion of male sticklebacks maintaining nests of different qualities ranging from 0 (low nest quality) to 2 (high nest quality) in relation to individual MHC class IIB allele diversity. See text for details on how nest quality was assessed. Sample sizes are given at the bottom of the bars.

Male breeding colouration

Data of the two food groups were pooled because an analysis of covariance showed no statistical difference between them ($N = 73$, $df = 1$, $F = 0.92$, $P = 0.341$). We found a strong positive correlation of male breeding colouration with individual MHC class I diversity, i.e. the higher a male's number of MHC class I alleles the redder it is (ANCOVA, $N = 73$, $df = 1$, $F = 9.29$, $P = 0.003$, Fig. 4). Due to the hypothesis of the two opposing selective forces acting on the number of MHC alleles – increased pathogen recognition versus increased auto-reactivity and thus increased de-selection of T-cell clones - we expected the males carrying an intermediate rather than a high number of MHC class I alleles to express the most intense colouration. However, this assumes the two selective forces to act on a purely quantitative basis, i.e. the number of alleles being selected regardless of the nature of the respective alleles. If there is, however, an allele providing strong resistance to a predominant pathogen, one could imagine a net benefit of having this allele even in individuals with a super-optimal

allele number. Since the likelihood to possess a specific allele should increase with the number of alleles per individual, we suspect that the males that are redder having a high diversity of MHC class I might carry such alleles. To test this hypothesis and to potentially identify such specific alleles, we compared the reddest quarter of the test fish with the dullest quarter according to their MHC class I allele composition. Two alleles were found to be present significantly more often in red males than in dull males. 65% of all red males carried Allele 16, while it was only 20% of the dull males (Chi-square test, $N = 40$, $df = 1$, $\chi^2 = 8.29$, $P = 0.004$); Allele 31 was present in 60% of the red males as opposed to 20% of the dull males (Chi-Square test, $N = 40$, $df = 1$, $\chi^2 = 5.01$, $P = 0.025$). Interestingly, when the data set was split up with respect to the two food groups, significance only remained in the poorly fed fish group (two-tailed Fisher's Exact Test, Allele 16: low food: $N = 18$, $P = 0.009$, high food: $N = 22$, $P = 0.395$; Allele 31: $N = 18$, $P = 0.0498$, high food: $N = 22$, $P = 0.67$).

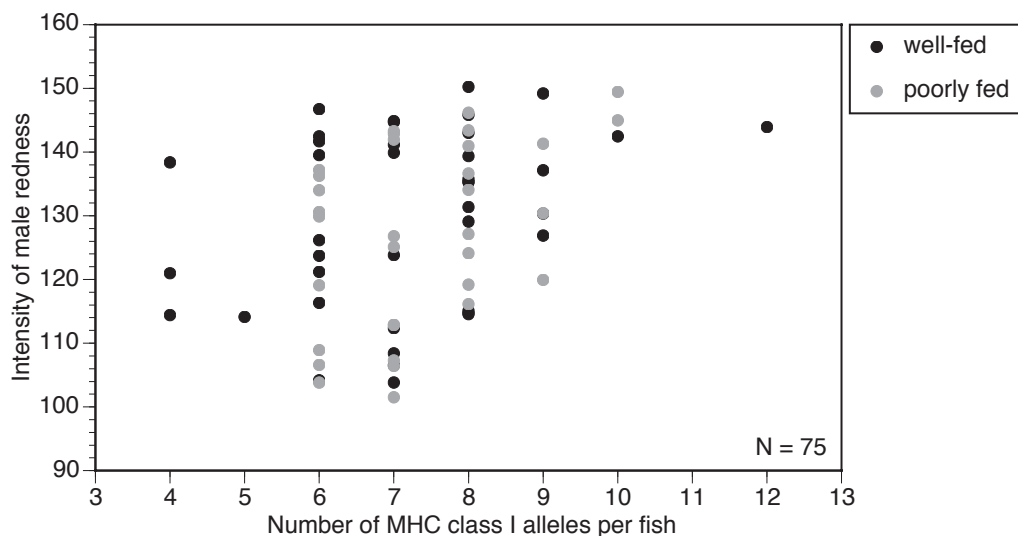


Figure 4: *Intensity of male breeding colouration in relation to individual MHC class I allele diversity.*

Within all test fish, the presence of at least one of these alleles was related to a more intense colouration (ANOVA, $N = 80$, $F_{2, 77} = 3.68$, $P = 0.03$, Fig. 5). Again, after splitting up the data set, this relationship only remained significant for the fish under low food conditions (ANOVA, low food: $N = 37$, $F_{2, 34} = 3.44$, $P = 0.044$; high food: N

= 43, $F_{2,40} = 0.794$, $P = 0.459$). Furthermore, for both alleles, the likelihood for an individual to carry them increased with increasing individual allele number (Weighted linear regression, $N = 165$, Allele 16: $r^2 = 0.83$, $F_{1,7} = 35.39$, $P = 0.0006$; Allele 31: $r^2 = 0.61$, $F_{1,7} = 10.96$, $P = 0.013$). It thus seems that certain alleles can indeed achieve such an importance that the advantage of carrying it outweighs the disadvantage of being above the optimal allele number. Various studies have found specific MHC alleles associated with resistance to a certain pathogen (e.g. Langefors *et al.* 2001, Grimholt *et al.* 2003, Bonneaud *et al.* 2005; reviewed in Milinski 2006) and also in the stickleback, four MHC class IIB alleles were found that were significantly associated with the resistance against one specific macroparasite species (Wegner 2004). It seems likely that the two MHC class I alleles that correlate with intense breeding colouration provide resistance against a specific and presumably common pathogen. Since MHC class I is involved in the recognition of intracellular pathogens such as viruses, identification and quantification of the pathogens was not feasible. Male sticklebacks showing a higher MHC based resistance could have a lower demand for innate immune activity, thus be less prone to oxidative stress (von Schantz *et al.* 1999; Graham *et al.* 2005), and therefore be able to allocate the available antioxidants to the carotenoid-based breeding ornamentation (Brush and Reisman 1965) instead. Experimental evidence for the existence of such a trade-off comes from a study on blackbirds, in which males immunized with an antigen showed a significant decrease in the intensity of the carotenoid-based colour of their bill (Faivre *et al.* 2003). Also three-spined sticklebacks reduced their carotenoid-based red colouration after being exposed to parasites (Milinski and Bakker 1990). Furthermore, it has been shown that sticklebacks with an optimal number of MHC class IIB alleles show the lowest respiratory burst reaction, an innate immune mechanism that generates reactive oxygen molecules to kill pathogens (Kurtz *et al.* 2004). Moreover, sticklebacks with a non-optimal MHC class IIB diversity have higher levels of MHC gene expression (Wegner *et al.* 2006), which correlates with increased oxidative stress (Kurtz *et al.* 2006). Hence, the above finding of males being redder when carrying at least one of the MHC class I alleles could be explained by the lower demand of radical scavengers due to decreased oxidative stress. The importance of the trade-off might, however, depend on the energy income of the fish as indicated by the fact that the allele associations only remained significant in the poorly fed group. As already

discussed for the females, well-fed males that do not carry at least one of those specific alleles might still be able to compensate a higher innate immune response and nevertheless become red, whereas poorly-fed males might not.

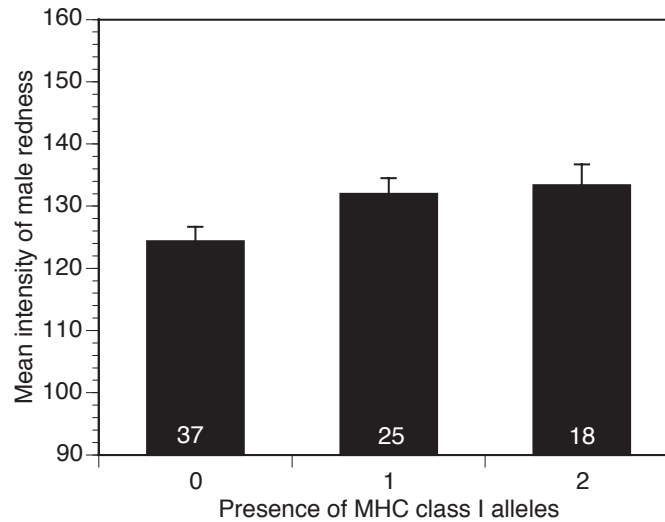


Figure 5: Mean intensity of male breeding colouration in relation to the presence of none (0), one (1) or both (2) of the identified specific MHC class I alleles. The two alleles were found to be carried significantly more often by individuals belonging to the reddest quarter when compared to the dullest quarter of the test fish. Sample sizes are given at the bottom of the bars. Error bars indicate one standard error.

In conclusion, although just correlative, our study underlines the general importance of MHC allele diversity with respect to individual fitness. It does not only support the hypothesis of an allele optimum for MHC class IIB, which for the three-spined stickleback is at about six alleles, it also, for the first time, finds MHC class I diversity to be related to individual fitness. Two MHC class I alleles were identified potentially providing resistance to a specific and presumably common pathogen, possibly explaining the unexpected finding of a maximal instead of an optimal allelic diversity with relation to male breeding colouration. It has previously been shown that the red throat is an honest signal revealing a male's health (Milinski and Bakker 1990). Our findings might take this a bit further in that not only a male's condition but also the genetic value of its immune system is expressed by the intensity of the red ornamentation supporting the good-genes hypothesis (Hamilton and Zuk 1982). Females choosing the reddest male would then not only gain fitness benefits through a

healthy mating partner taking care of the offspring, but also by ensuring that the offspring will be endowed with a good immune system. This is in accordance with the finding that offspring of brighter males were more resistant to infections with the cestode *Schistocephalus solidus* (Barber *et al.* 2001a). Furthermore, when redness can be linked to resistance against presumably common pathogens, olfactoric mate choice based on MHC allele number could be improved by visual mate choice guaranteeing that not only the optimal MHC allele number but also relevant resistant alleles are provided for the offspring as had been suggested by Aeschlimann *et al.* (2003) and Milinski (2006).

Conclusion

Half of the existing species are parasites (Price 1980; May 1988), and there is probably no organism without parasites (Poulin 1996b). They occur in an extremely large variety and their capability of changing and adapting is remarkable. Parasites are therefore considered to be one of the major driving forces in the evolution of organisms (Hamilton 1980; Ebert 1994). In this thesis, I present four studies using the three-spined stickleback and its cestode parasite *S. solidus* as model organisms that enlighten co-evolutionary aspects of parasites and their hosts. In this interplay, parasite-parasite interactions are probably as important as host-parasite interactions. Studies on both are needed if we want to understand evolution and predict dynamics, which are particularly important for designing drug treatments and managing epidemics.

Hosts are frequently multiply infected and the specific within-host dynamics have important consequences for the outcome of the infection. Interactions between individuals of *S. solidus* co-infecting the stickleback could be influenced by their mating decision since they are likely to be future mating partners in the final bird host. Surprisingly, despite high inbreeding depression, *S. solidus* was found to prefer a related mating partner over an unrelated one. Selective forces leading to this unexpected preference might be indirect fitness benefits as predicted by kin-selection theory, a decrease in sexual conflict since related individuals might be less likely to cheat on each other, or purging the genetic load. The three potential forces are not mutually exclusive and emphasise the complexity concerning the selective pressures involved. Infecting sticklebacks with either two related or two unrelated individuals of *S. solidus* led to significantly more related double infections. This suggests that infection intensities are higher in genetically uniform infections. In sequential double infections, the secondly acquired parasite had a competitive advantage compared to the first one. One explanation for this unexpected result might be that the first cestode promotes a more favourable environment for the second one by suppressing the stickleback's immune system and paying the costs alone. This result suggests that host-parasite interactions are tightly connected with parasite-parasite interactions. In response to high parasite pressure, hosts are expected to be endowed with an effective immune system. With respect to *S. solidus* infections, the stickleback's innate immunity appears to be very important. The major histocompatibility complex

(MHC) plays a key role in adaptive immunity of vertebrates and stickleback fitness traits were significantly influenced by individual MHC allele diversity. While an expected optimal MHC class *IIB* allele diversity was found in males maintaining a high quality nest, male breeding colouration was most intense at a maximal MHC class I allele diversity. Two MHC class I alleles were identified to be associated with a higher intensity of red colouration. They were also more likely to be carried at a higher individual allelic diversity, which potentially explains the positive correlation. It seems likely that these two alleles confer resistance to a specific and presumably common parasite. Thus, resistance might not only be a matter of allele quantity but also of allele quality.

The studies presented in this thesis have generated several unexpected results. Interactions have been shown to be very complex and multilayered, which underlines the importance of further investigating parasites and their hosts to gain a deeper understanding of their evolution and dynamics.

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seit November 2002:	Promotion am Max-Planck-Institut für Limnologie, Plön

Publikationen:

Schuster, S., Rossel, S., Schmidtman, A., Jäger, I., Poralla, J. (2004). Archer Fish learn to compensate for complex optical distortions to determine the absolute size of their aerial prey. *Current Biology* **14**, 1565-1568.

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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. Teile dieser Arbeit wurden als Manuskripte bei Zeitschriften eingereicht und veröffentlicht: Kapitel II wurde mit Solveig Schjørring als Koautorin in *Evolution* veröffentlicht. Die restlichen Kapitel befinden sich in Vorbereitung für eine Publikation: Kapitel I mit Solveig Schjørring als Erstautorin; Kapitel III mit Joachim Kurtz, Martin Kalbe und Jörn P. Scharsack als Koautoren und Kapitel IV mit C. Kerstin Krobbach, Helmut Schaschl, Thorsten B. H. Reusch, Martin Kalbe, Siân Griffiths und Manfred Milinski als Koautoren. Die einzelnen Studien wurden in Kollaboration mit den oben aufgeführten Mitautoren durchgeführt. Dabei war mein Beitrag wie folgt: Kapitel I: Durchführung und Auswertung der genetischen Analysen; Kapitel II: Durchführung, Auswertung und Erstellen des Manuskripts; Kapitel III: Durchführung, Auswertung und Erstellen des Manuskripts; Kapitel IV: Auswertung und Erstellen des Manuskripts.

Plön, 25. April 2006

Ilonka Jäger