Host parasite interactions in a cestode with a complex life cycle, *Schistocephalus solidus*

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Katrin Hammerschmidt

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Summary

Many parasites have complex life cycles, i.e. they have to pass through several host species to reach maturity. Hence complex life cycles often consist of invertebrate and vertebrate hosts, the parasite likely varies in the machinery required for infection, exploitation and transmission of each host. Does the ability to optimally exploit one host inevitably lead to a reduced ability for the parasite to exploit another host in its life cycle? To answer this question, I analysed parasite life history traits like transmission, infection, and establishment in the model system of the tapeworm *Schistocephalus solidus* in relation to its two intermediate hosts, a cyclopoid copepod, and the three-spined stickleback. In this thesis, I particularly focus on interactions with the hosts' immune systems and on constraints, which are potentially shaping the evolution of virulence in parasites with complex life cycles.

The first difficulty for a parasite in a complex life cycle, compared to a single host system, is to successfully manage the additional transmission steps between hosts. Orally transmitted parasites often depend on predation of the current host by the next host. Therefore, to enhance transmission probability, parasites would profit from increased conspicuousness of the current host, at the time when the parasite is ready for transmission to the next host. In this thesis I detected that with *S. solidus*, infected copepods became more active and that they stored higher amounts of orange carotenoid droplets. They thus increased in conspicuousness when the parasite was ready for transmission to the visually hunting three-spined stickleback (chapter I and II).

After a parasite successfully found and orally entered the next host, an important step is the penetration of the intestinal mucosal wall. Individuals of *S. solidus* are eaten within copepods by its second intermediate host, the three-spined stickleback, and subsequently penetrate the anterior part of the midgut within 14 to 24 hours. Contrary to previous assumptions, I found that the outer body layer of *S. solidus*, together with the cercomer, is already lost in the stomach of the stickleback so that the underlying tegument with its microtriches is exposed. This most probably plays an important role in migration into the body cavity (chapter III).

In each host, parasites have to survive the encounter with the host's immune system. Carbohydrates on the parasite's surface are relevant to mediate host non-self recognition and parasite camouflage. Evidence in this thesis suggests that

carbohydrates are also important in *S. solidus*, hence I found individual tapeworms to change their surface when switching from the invertebrate to the vertebrate host. Among individual parasites the variation in surface sugar composition was linked to parasite fitness parameters in the second intermediate host (chapter IV; Hammerschmidt & Kurtz 2005b).

If parasites with complex life cycles cope better with one of the different types of host immune systems, the parasite should perform differently in the other hosts. I found, that parasite sibships of *S. solidus* traded off adaptation towards different parts of their hosts' immune systems. Sibships that performed better in the invertebrate host also induced lower levels of activation of innate immune components and were less virulent in the fish host. Above all, this substantiates the constraint of both hosts' immune systems on parasite performance and the impact on evolution of virulence in a parasite with a complex life cycle (chapter V; Hammerschmidt & Kurtz 2005a).

Zusammenfassung

Viele Parasiten haben komplexe Lebenszyklen, d.h. sie müssen mehrere Zwischenwirte durchlaufen, um zur Geschlechtsreife im Endwirt zu gelangen. Sowohl Invertebraten als auch Vertebraten sind häufig als Wirte Bestandteil komplexer Lebenszyklen. An diese unterschiedlichen Wirtstypen müssen sich die Parasiten anpassen. Es ist daher wahrscheinlich, dass sich die Parasiten von Wirt zu Wirt in der Art der Infektion, Ausbeutung und Übertragung unterscheiden. Führt die Fähigkeit eines Parasiten, einen bestimmten Wirt optimal auszubeuten notwendigerweise zu einer verminderten Fähigkeit bei der Ausbeutung eines weiteren Wirtes aus seinem Zyklus? Zur Beantwortung dieser Frage untersuchte ich Merkmale im Lebenskreislauf eines Parasiten, wie den Erfolg der Übertragung, Infektion und Etablierung in dem Modellsystem des Fischbandwurms *Schistocephalus solidus* und seinen zwei Zwischenwirten, einem cyclopoiden Ruderfußkrebs (Copepoden) und dem dreistachligen Stichling. In meiner Doktorarbeit konzentriere ich mich besonders auf die Interaktionen des Parasiten mit den verschiedenen Immunsystemen der Wirte und auf mögliche Zwänge, die die Evolution von Virulenz in komplexen Lebenszyklen beeinflussen könnten.

Im Vergleich zu Wirt-Parasit Systemen mit nur einem Wirt, müssen Parasiten in komplexen Lebenszyklen unterschiedliche Schritte bei der Übertragung zum nächsten Wirt meistern. Parasiten, die oral übertragen werden sind z.B. davon abhängig, dass der zukünftige Wirt den jetzigen Wirt frisst. Wenn der Parasit zur Übertragung bereit ist, könnte seine Übertragungswahrscheinlichkeit dadurch erhöht werden, dass sein aktueller Wirt genau zu diesem Zeitpunkt für den zukünftigen Wirt auffälliger (attraktiver) würde. In zwei Experimenten dieser Doktorarbeit konnte ich zeigen, dass mit *S. solidus* infizierte Copepoden aktiver waren und auch mehr orangene Karotinoidtröpfchen einlagerten als nicht-infizierte. Diese Veränderungen traten jeweils erst dann ein, wenn der Parasit für den nächsten Wirt, den visuell jagenden dreistachligen Stichling, infektiös geworden war (Kapitel I und II).

Für die meisten Parasiten, die nach der Wirtsfindung von diesem oral aufgenommen werden, ist der nächste kritische Schritt, das Erreichen und Penetrieren der Darmwand, um in andere Gewebe des Wirtes zu gelangen. In diesem Zusammenhang konnte ich zeigen, dass das zweite Larvenstadium (Procercoid) von *S. solidus* innerhalb von 14 bis 24 Stunden nachdem sie zusammen mit den

Copepoden gefressen wurden, den vorderen Mitteldarmteil des Stichlings penetrierten. Ergebnisse in Kapitel III dokumentieren, dass im Gegensatz zu bisherigen Annahmen, die äußere Körperhülle von *S. solidus* schon im Fischmagen verloren geht. Auf diese Weise wird das darunterliegende Tegument mit den Microtriches freigelegt, welches eine wichtige Rolle bei der Migration in die Körperhöhle spielen könnte.

Einmal im Wirtskörper angekommen, müssen Parasiten außerdem die Abwehrreaktionen der Immunsysteme überleben. Es ist bekannt, dass Kohlenhydrate an der Parasitenoberfläche die Fremderkennung des Wirtes und die Tarnung des Parasiten steuern. In Kapitel IV dieser Doktorarbeit konnte ich unter anderem zeigen, dass Kohlenhydrate auch bei *S. solidus* eine wichtige Rolle spielen. Die Bandwurmlarven änderten ihre Kohlenhydrat-Oberfläche, wenn sie von dem Invertebraten (Copepoden) zu dem Vertebraten Wirt (Stichling) wechselten. Individuelle Unterschiede in der Zuckerzusammenstellung auf der Parasitenoberfläche konnten mit Fitnessparametern des Parasiten im zweiten Zwischenwirt korreliert werden (Hammerschmidt & Kurtz 2005b).

Es könnte durchaus sein, dass ein Parasit, der mit dem Immunsystem eines seiner Wirte besonders gut zurechtkommt, schlechter an die Immunsysteme der anderen Wirte angepasst ist. Das ist besonders in Hinblick auf einen Wirtswechsel zwischen Invertebraten und Vertebraten interessant, da erstere nur ein unspezifisches Immunsystem, letztere aber zusätzlich auch noch ein spezifisches Immunsystem haben. In Kapitel V zeige ich, dass *S. solidus* Geschwisterschaften die Anpassung an die verschiedenen Teile der Immunsysteme der Wirte gegeneinander abwägen. Die Geschwisterschaften, die besser im wirbellosen Wirt zurechtkamen, riefen im Vertebraten eine geringere Aktivierung des unspezifischen Immunsystems hervor und führten in diesem Wirt zu weniger Schaden, d.h. waren weniger virulent. Dieses Ergebnis macht besonders deutlich, dass die Interaktion des Parasiten mit den Immunsystemen der verschiedenen Wirte nicht nur den Erfolg des Parasiten beeinflusst sondern auch die Evolution von Virulenz in einem Parasiten mit einem komplexen Lebenszyklus erklären kann (Hammerschmidt & Kurtz 2005a).

Introduction

Host parasite interactions

More than half of all plant and animal species are parasites (Price 1980), and there is probably no organism without any parasites (Poulin 1996). Parasitism is defined as the case in which one partner, the parasite, of an interacting species pair is dependent upon the other interacting species, the host, for survival (MacInnis 1976). Both, parasites and hosts, have evolutionary effects on each other, resulting in coevolution, which is defined as the process of reciprocal adaptive gene change in two or more species (Woolhouse et al. 2002). Parasites may evolve in a way to maximize host exploitation, whereas hosts may evolve mechanisms to defend themselves against parasites. After a host has evolved a good defence against parasites, natural selection may act again on the parasite to overcome this defence, and counter-adaptations will evolve. This potentially results in a continuous arms race of adaptations and counter-adaptations in several traits on both sides, e.g. host seeking versus parasite avoidance, parasite infectivity versus host resistance, and evasion of host defence systems versus clearance of infection (Figure 1).

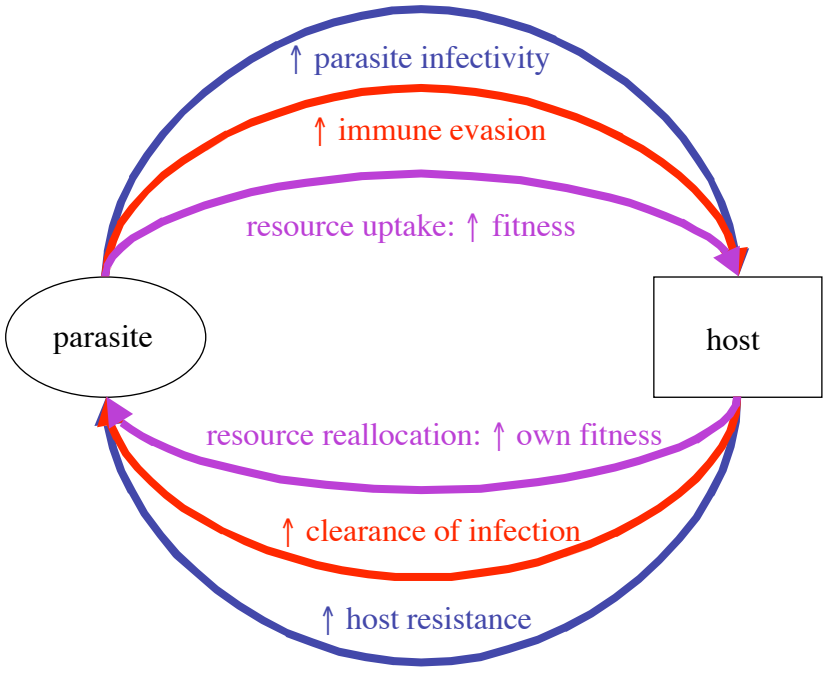


Figure 1: Host parasite coevolution in a single host parasite system.

Such a continuous arms race does not have to be unidirectional but potentially results in a constant cycling of genotype frequencies in the host and parasite population, known as the Red Queen dynamics. Van Valen (1973) cited a remark from 'Through the looking glass' (Carroll 1871), where the Red Queen says to Alice: "Now, here you see, it takes all the running you can do, to keep in the same place" to describe coevolution between biological enemies, like predator - prey or host - parasite. This illustrates the continuous arms race in a continuously changing environment where the organisms interact as expected under time-lagged, frequency-dependent selection (Lively 1996). The Red Queen hypothesis might explain observations in nature such as the maintenance of genetic polymorphism, the evolution of virulence but is best known to explain the existence of sex (Woolhouse & Webster 2000). Here, the basic idea is that genetic diversity of host populations is maintained by sexual recombination hindering the parasite to optimally adapt to the hosts, even though parasites, due to shorter generation times and larger numbers, are assumed to be ahead in the coevolutionary arms race (Ebert & Hamilton 1996; Hamilton 1980).

Models of host parasite coevolution could potentially be applied in medical science, e.g. for development of drugs or vaccines or for predicting host and parasite dynamics in natural populations. Unfortunately this tool can be only used for few parasite species, since nearly all parasites of medical and veterinary relevance have complex life cycles, i.e. they have to pass through several host species to reach maturity (Poulin & Cribb 2002, Parker 2003a).

Host parasite interactions in complex life cycles

Compared to a single-host system, where the parasite only has to adapt to one host (Figure 1), the process of coevolving with at least two hosts in a complex life cycle (Figure 2) is much more difficult and not as well understood. In a recent theoretical study, Gandon (2004) points out that the different hosts can be seen as different environments that a parasite has to adapt to. He proposes the machinery required for transmission, infection and exploitation and thus the selection pressures on the parasite, to vary depending on each host. He further predicts the evolution of constraints among different parasite traits, both within and between different hosts that influence the evolution of virulence.

In the following, I will therefore focus on the main steps, a parasite has to accomplish while completing its complex life cycle, namely the transmission between the different hosts, and the interaction with the variable hosts' immune systems. In particular, I will link the constraints between the different steps to the evolution of virulence in complex life cycles.

Compared to a single host parasite system, a multi-host life cycle is more difficult to complete due to several improbable transmission steps from one host to the next (see also Figure 2) (Poulin & Cribb 2002). Selection pressure on increasing transmission probability is expected to be high for the parasite, whereas selection pressure on increasing behavioural resistance should be high for the host. The idea that a parasite could potentially enhance transmission probability between hosts but also that the host could avoid eating infected prey, has fascinated scientists since in the early 20th century (Cram 1931; reviewed in Thomas et al. 2005).

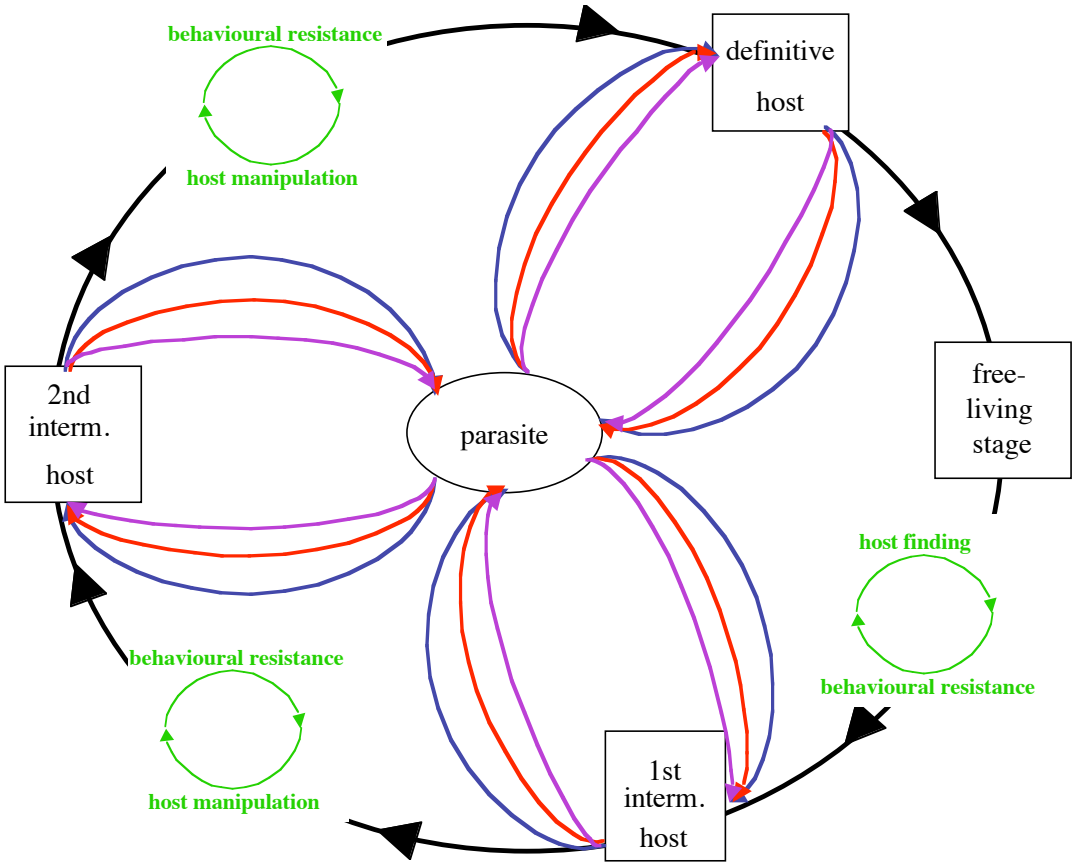


Figure 2: Host parasite coevolution in a parasite with a complex life cycle. The parasite has to adapt to each of the three hosts.

Increased transmission through host manipulation

Parasite-induced alterations of various host phenotypic traits that enhance transmission probability, like morphology, physiology and behaviour have been reported for a wide range of parasites (Moore 2002; Poulin 1998). In particular, the focus of many experimental studies has been whether adaptive parasite-altered host behaviour (host manipulation) exists, that enhances parasite fitness (reviewed in Thomas et al. 2005).

Three major problems arise when studying host manipulation (Poulin 1995). First, it is difficult to differentiate whether a phenotypic change of the host is adaptive for the parasite or whether it is a non-adaptational pathological by-product of infection, e.g. decrease in host activity due to resource depletion by the parasite. Second, behavioural changes could also occur as an adaptation of the host to reduce fitness costs of infection, e.g. decrease in host activity is caused by reallocation of resources to reproduction. Third, it could also be a phylogenetic constraint, inherited from an ancestor, which still leads to a selective advantage in the present host parasite system. Poulin (1995) formulated 'guidelines' to distinguish between the various possibilities: to consider a behavioural change as an adaptive behavioural manipulation, it has to meet several criteria, like complexity, 'purposive design', independent evolution in several host and parasite lineages and fitness increase for either the host or the parasite. Nevertheless, the different hypotheses of parasite manipulation, by-product of infection or fitness-cost reduction do not need to be mutually exclusive (McCurdy et al. 1999; Poulin 1994a) but should also not be the only focus of research in this area (Thomas et al. 2005). In several recent publications, it is even stressed that other aspects in that area are considered to be of equal or higher relevance, e.g. understanding the mechanisms and analysing a larger number of phenotypic traits to understand parasitized hosts as deeply modified organisms with several modifications, some of which may favour parasites and some which may favour hosts (Cézilly & Perrot-Minnot 2005; Thomas et al. 2005; Webster 2005).

When a next host has successfully been 'found', the parasite has to adapt to a completely new environment. Many complex life cycles consist of several

invertebrate and vertebrate hosts, all with different physiologies and particularly different immune systems the parasite has to adapt to.

Some basics about parasite recognition by host defence systems

The immune system of the host is responsible for parasite recognition, and is traditionally differentiated into innate and adaptive immunity. Janeway et al. (1999) define innate immunity as the early phase of the host response to infection in which a variety of innate mechanisms recognize and respond to the presence of a pathogen. In contrast to adaptive immunity, innate immunity is present at all times, does not increase with repeated exposure to a given pathogen, and does not discriminate between pathogens. Adaptive immunity, also known as acquired immunity, is the response of antigen-specific lymphocytes to antigen, including the development of immunological memory.

The invertebrate immune system consists of innate immunity, whereas the vertebrate immune system comprises of both, innate and adaptive mechanisms. Recently, evidence for the existence of specific immunological recognition and moreover of specific memory in the innate immune system of invertebrates is accumulating, challenging the dogma that specific recognition and immunological memory is confined to the adaptive immune system (Janeway et al. 1999; Kurtz 2005; Litman et al. 2005).

The mechanisms leading to specificity in the invertebrate immune system are, as yet, not clear. However, specificity in innate immune systems is not based on the mechanisms that mediate specificity in vertebrates, i.e. diversified lymphocyte receptors and antibodies (Klein 1989). Recent studies allude to the recognition of carbohydrate residues, probably by lectin-like proteins as mediators of specific recognition and memory in invertebrates (reviewed in Kurtz 2005; Zhang et al. 2004). Pathogen-associated molecular patterns (PAMPs), mainly carbohydrate residues, can be bound by peptidoglycan recognition molecules and so invading microbes be recognized (Guan et al. 2004; Janeway et al. 1999; Salzet 2001). These components of the innate immune system are highly conserved and are present in both, invertebrates and vertebrates.

After a parasite has successfully entered and established itself in the host, it starts exploiting the hosts' resources and thus causes irrevocable damage, often death. Why do parasites kill their hosts? Intuition suggests that parasites should evolve to be benign as long as the host is needed for survival, e.g. until transmission to the next host is accomplished (Bull 1994). The question, why natural selection may favour virulent parasites over avirulent ones, is still unsolved and puzzles many scientists in that field. Several models were designed to deal with this problem, but experimental evidence is, as yet, scarce. Aside from an increasing academic interest, the evolution of virulence is probably the most important topic in evolutionary parasitology in the face of its medical and agricultural applications, e.g. designing better vaccines or prevent emergence of highly virulent strains.

Virulence

Virulence is defined as the amount of damage a parasite causes to its host, thereby reducing the host's fitness (Frank 1996). Evolutionary theory considers virulence as a consequence of parasites adapting to a natural host population in order to maximize fitness (Imhoof & Schmidt-Hempel 1998). In many theoretical models, virulence is therefore assumed to be correlated with other life-history traits of the parasite, like production of transmission stages (May & Anderson 1990). During host exploitation, the parasite faces a life-history trade-off between fecundity (production of transmission stages), which ideally should be high, and host longevity (often inversely related to damage to the host), which ideally should be long. Other trade-offs among components of parasite fitness can also influence virulence, e.g. competition among parasite genotypes favouring faster growth of one genotype over the others and can rapidly lead to overexploitation of the host (Frank 1996).

Many parasites of medical importance have a multi-host life cycle, e.g. all vector-borne diseases, like *Plasmodium* that causes malaria or helminth parasites, like cestodes or nematodes. For the evolution of virulence in parasites with complex life cycles, more factors need to be taken into account, as stressed by Gandon (2004). In the theoretical study he points out, that virulence is not only shaped by trade-offs between life-history traits of the parasite within one host but moreover by trade-offs between life history traits between the different hosts. A

parasite that has to adapt to two types of hosts most probably needs different machineries for host exploitation. If the optimal exploitation of one type of host (adaptation to the host) is associated with a reduced ability to exploit another host, it could result in negative genetic covariances (trade-offs) between traits. This was nicely shown by Davies et al. (2001), one of the few empirical studies in this field, who observed a trade-off between the reproductive success of schistosomes in their mammalian and molluscan hosts.

Only few studies so far considered physiological factors, like host immunity as a selection pressure on virulence. One important study dealing with this showed that sibships of *Plasmodium chabaudi* became more virulent when they evolved in immunized, as compared to naïve, mice (Mackinnon & Read 2004). In multi-host life cycles, parasites may be forced to trade-off between different immune evasive strategies and potentially lead to a divergent activation of the immune systems. How that shapes and constrains evolution of virulence is, as yet, not clear.

Schistocephalus solidus, a parasite with a complex life cycle

There are several reasons why the tapeworm *Schistocephalus solidus* and its hosts are a very good model system to study host parasite interactions, specifically in complex life cycles. Generally, the maintenance, culture and manipulation of all life cycle stages in the laboratory was established more than 50 years ago and has been constantly improved since then (Dubinina 1966; Smyth 1946; Wedekind 1997). During this time, a great amount of knowledge about host parasite interactions in this system was gathered. Hosts and parasites are easily caught in the wild, so that host parasite interactions can be studied in a naturally co-evolved host parasite system. This is a huge advantage over many other host parasite model systems, which either consist of non-natural hosts or hosts that are adapted to laboratory conditions for many generations.

S. solidus has to pass through three hosts before finally reproducing in any species of fish-eating bird. When eggs are released into freshwater with the birds' faeces, coracidia, the first, free-swimming larval stage, hatch, and are preyed upon by various species of freshwater cyclopoid copepods. After reaching the gut of the copepod, a coracidium looses its outer shell, and bores, as an oncosphere, through the gut wall into the body cavity, where it transforms into a procercoid larva. It grows in this first intermediate host (in this system the copepod *Macrocyclops albidus*) until it is infective to the second intermediate host, specifically the three-spined stickleback, *Gasterosteus aculeatus*. Here, it grows as plerocercoid in the body cavity until it is infective to the final host, any fish eating bird. In the laboratory, we replaced the definitive host, the bird, with an *in vitro* breeding system (Smyth 1946; Wedekind 1997).

As in many other complex life cycles, *S. solidus* has to adapt to very different hosts, more precisely to an invertebrate and two vertebrate hosts. Based on theoretical predictions (Gandon 2004), constraints between parasite life-history traits are expected to result in difficulties of the parasite to adapt to all three hosts equally well. In our system, we can imagine several traits of the parasite (and hosts) to be under selection pressure, specifically transmission between the hosts, and the process of infection and establishment, especially evading the hosts' immune systems.

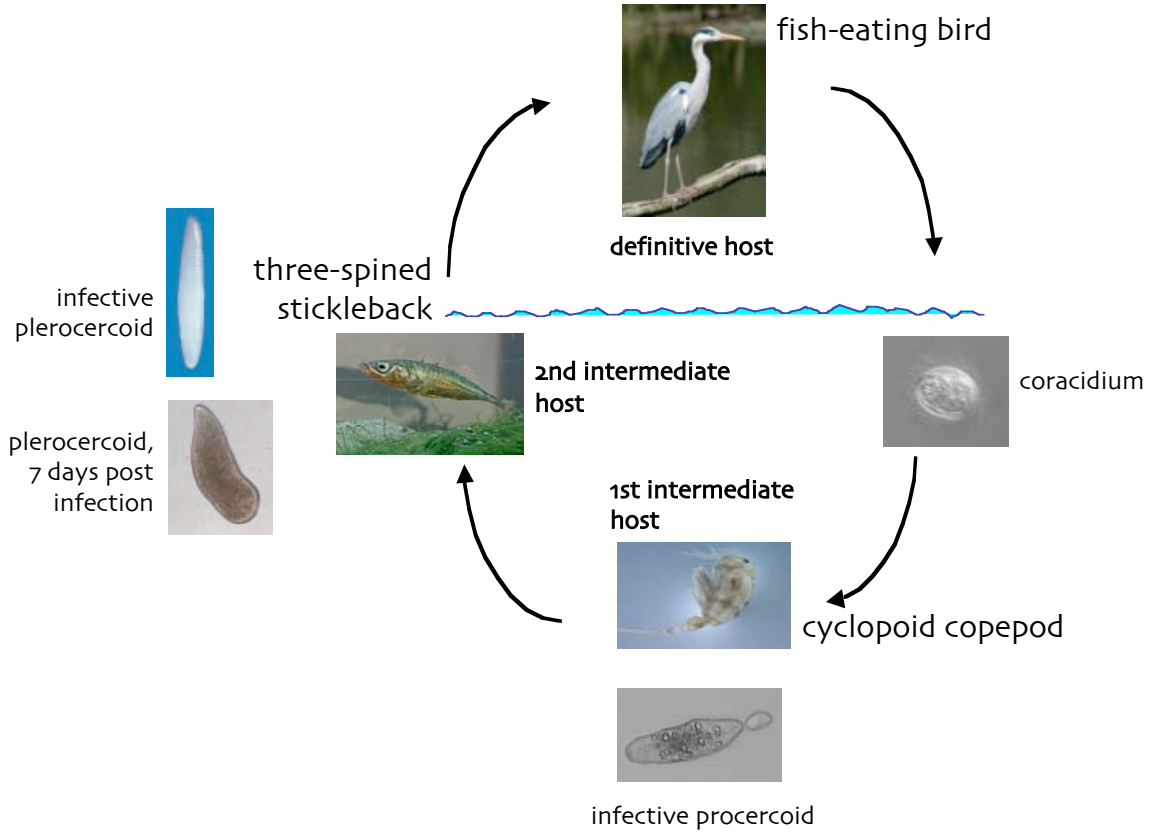


Figure 3: Life cycle of the cestode *Schistocephalus solidus*.

To study these aspects of host parasite coevolution in this system, one should focus on the intermediate hosts, since the major uptake of resources takes place in the two intermediate hosts, which leads to serious fitness reductions for both hosts (Arme & Owen 1967; Wedekind 1997). In contrast, the final host, any fish-eating bird, is most probably not strongly influenced by the presence of the tapeworm in the gut, since the parasite does not take up any resources and stays for only a few days (Dubinina 1966). Selection pressure on host defences is thus thought to be highest for the two intermediate hosts, the copepod and the stickleback.

Increased transmission probability can generally be achieved by increasing the intermediate hosts' attractiveness as prey (Bakker et al. 1997). Infection with *S. solidus* has been found to lead to several behavioural changes in both intermediate hosts, like decrease of escape ability, changes in microhabitat choice or reduced predator avoidance (Jakobsen et al. 1988; Jakobsen & Wedekind 1998; Milinski 1990; Wedekind & Milinski 1996). However, none of the studies so far, either excluded initial quality differences between infected and non-infected hosts or

measured changes in several host traits. However, host manipulation is likely to include more than one trait and should only start when the parasite is ready for transmission to the next host (Thomas et al. 2005). In chapter I and chapter II of this thesis, I will therefore analyse if important host traits, like activity and colouration, change during infection and investigate whether changes in behaviour and host appearance only occur when the procercoid stage of *S. solidus* is ready for transmission to the stickleback host.

After the parasite successfully 'finds' a host, the next phase in the arms race of attack and defence starts: the parasite has to enter the body cavity and get established in its final location within the hosts' body. In the first intermediate host, the time-period between ingestion of the parasite and the penetration of the gut wall of the copepod was shown to be of crucial importance in determining the outcome of infection (van der Veen & Kurtz 2002). As yet, not much is known about the infection process of the second intermediate host, the stickleback. To determine the selective forces acting on the parasite at that stage, the process of infestation of the three-spined stickleback will therefore be analysed in chapter III of the thesis.

Once in the hosts' tissues, the parasite has to evade the host's immune system. This is particularly interesting in the *S. solidus* system, since interactions with the immune systems of both intermediate hosts, an invertebrate and a vertebrate, have been observed in previous experiments. The copepod host was found to possess specific memory against tapeworm larvae of *S. solidus* (Kurtz & Franz 2003), and in the stickleback host, indications for suppression of the immune system were found (Scharsack et al. 2004). One aim of the thesis (chapter IV) is to investigate experimentally if carbohydrates on the tapeworm surface could be involved in immune-evasion in the vertebrate host. In this context, it is especially interesting if the interaction with the two different immune systems in both intermediate hosts potentially leads to constraints in parasite performance and virulence (chapter V).

Thesis outline

The thesis is divided into six chapters. Each chapter represents an independent study that is structured into abstract, introduction, methods, results and discussion. Thereby various aspects of host parasite interactions in the life cycle of *Schistocephalus solidus* are addressed. This outline gives a short overview of the motivation for each experiment.

Chapter I

In the first chapter I investigated if copepods, the first intermediate host of *S. solidus*, show transmission-enhancing behavioural alterations when infected. Therefore, copepods of the species *Macrocyclops albidus* were singly exposed to coracidia of *S. solidus* and host activity measured. Both, an increase or a decrease in activity could potentially increase transmission. One precondition for a direct host manipulation is, that the host should only change behaviour when the parasite has reached the infective stage for the next intermediate host, the three-spined stickleback. To test that and to exclude initial differences in host quality or allocation of resources to other life history traits, copepod activity was monitored pre exposure and at several points in time post exposure to parasites.

Chapter II

The aim of this experiment was to test whether copepods change their appearance, specifically the storage of carotenoid reserves, when infected with *S. solidus*. A decrease of carotenoids can be expected if they are used in the host's immune defence. The opposite, an increase in storage of carotenoids can be expected when the reserves are manipulated by the parasite. Larger carotenoid reserves make copepods more conspicuous, which probably leads to higher predation rates by visually hunting three-spined sticklebacks that are attracted by orange colours. It was tested if infected copepods differed from uninfected ones over time in the changes of carotenoid volume by measuring carotenoid reserves pre exposure and at several points in time post exposure to parasites.

Chapter III

After a parasite has entered a host it has to reach the final localization, within the host's body, where it becomes established. This phase is considered to be highly relevant in determining infection success in many parasite species. Therefore, I performed an experiment to investigate which factors determine host resistance and parasite infection success in *S. solidus* and its second intermediate host, the three-spined stickleback. After being ingested by the fish, the tapeworm has to reach the body cavity where it grows until it is infective for the next host. Using histological sections, I monitored the chronology of infection, the location of penetration into the body cavity, and the potential role of the different layers of the parasite's surface.

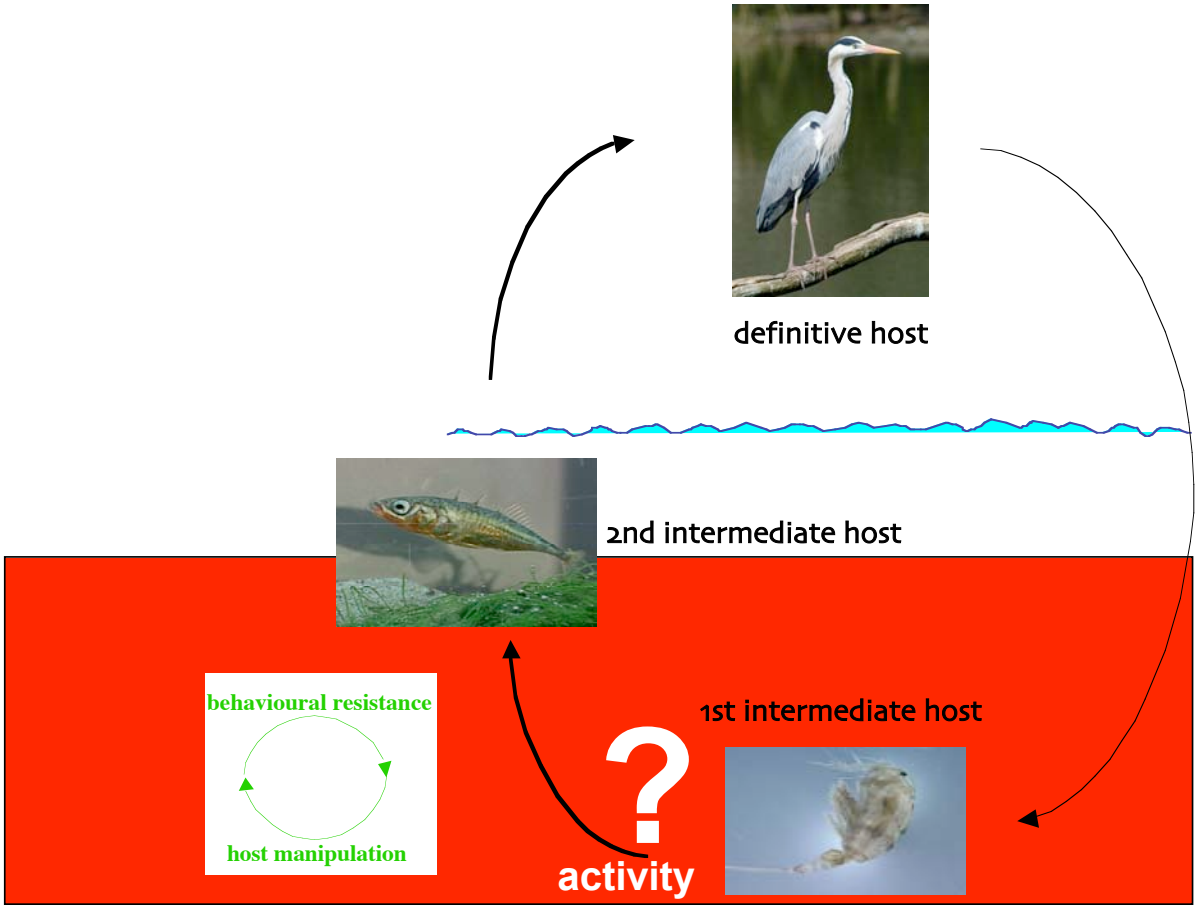
Chapter IV

Once in the hosts' tissues, parasites face the immune response of their hosts. Carbohydrates on parasite surfaces have been shown to play an important role in host non-self recognition and parasite camouflage. Because of the difference between invertebrate and vertebrate immune systems, I expected the different parasite stages to change surface carbohydrates upon host switch from the copepod to the stickleback host. To further explore fitness consequences of surface sugar composition, I used different parasite genotypes and related variation in surface carbohydrates to their performance (infectivity and growth) in the two hosts.

Chapter V

The divergent activation of hosts' immune systems in parasites with complex life cycles could potentially shape and constrain virulence. Multi-host parasites may be forced to trade-off between different immuno-evasive strategies. I therefore examined whether individuals of *S. solidus* are genetically constrained in their ability to evade the copepod and the fish equally. For this test, I used different genotypes of the tapeworms and analysed their performance in both hosts using infectivity and growth as fitness parameters. I further measured parameters of the stickleback immune response, since they were expected to mediate parasite performance.

CHAPTER I


definitive host
2nd intermediate host
behavioural resistance
host manipulation
1st intermediate host
?
activity

Copepod activity varies in the course of a parasite infection

Abstract

In a naturally occurring host parasite system with the tapeworm *Schistocephalus solidus* and its first intermediate host, the copepod *Macrocyclops albidus*, we investigated if copepods show transmission-enhancing alterations in activity when infected. For the altered activity being a direct host manipulation, the host should only change behaviour when the parasite has reached the infective stage for the next intermediate host, the three-spined stickleback. To test that and to exclude initial differences in host quality or allocation of resources to other life history traits, copepod activity was monitored pre exposure and at several points in time post exposure to parasites. In contrast to previous studies, we found that infected copepods decreased in activity. The significant difference in activity between exposed and control treatment started at 2 days post exposure, suggesting resource allocation to the hosts' immune systems. Activity declined even further in the infected group around day 5 and levelled off until the end of the experiment. Our findings are contradicting other studies on the same system, which stresses the complexity of the interactions between *S. solidus* and its copepod host and pointing towards an understanding of parasitized hosts as deeply modified organisms.

Introduction

Theoretical and empirical studies of host parasite coevolution increasingly focus on parasites with complex life cycles, i.e. parasites that have to pass through several hosts to reach maturity (Gandon 2004; Parker et al. 2003a, b; Poulin & Cribb 2002). Although adding steps to a life cycle seem to have many evolutionary advantages, these additional transmission steps between subsequent hosts are believed to be a major disadvantage for multihost parasites (Poulin & Cribb 2002). Adding another intermediate host, means adding another improbable transmission step, which more likely leads to a failure to complete the life cycle and consequently in no reproductive fitness at all.

Transmission-enhancing behavioural alterations of intermediate hosts due to infection with a parasite are well known in the literature and have been reported for a wide range of parasites (summarized in Moore 2002). Most studies in that field tried to differentiate whether parasite induced behavioural modifications of hosts were generated through actively manipulating parasites or were merely by-products of infection due to physiological damage caused by the parasite (Moore 2002; Poulin 1994a; summary in Poulin 1995).

One parasite that is known to cause behavioural changes in its hosts is the cestode *Schistocephalus solidus*, a horizontally transmitted parasite with a complex life cycle. It has to pass through two intermediate hosts, a cyclopoid copepod, such as *Macrocyclops albidus* and specifically the three-spined stickleback *Gasterosteus aculeatus* before reproducing in the gut of a fish-eating waterbird (Dubinina 1966). Transmission to the next host is facilitated through parasite induced behaviour modifications in both intermediate hosts (Arme & Owen 1967; Barber et al. 1995; Jakobsen & Wedekind 1998; Milinski 1985; Milinski 1990; Wedekind & Milinski 1996). In the stickleback, the tapeworm was, amongst others, found to lead to slow movements, decreased competitive ability, inefficient feeding and reduced predator avoidance (Arme & Owen 1967; Barber et al. 1995; Milinski 1985; Milinski 1990). Also in the copepod, many studies described changes in behaviour of infected animals, like decrease of escape ability, increase of general activity and changes in microhabitat choice (Jakobsen & Wedekind 1998; Urdal et al. 1995).

One study found that *S. solidus* infected copepods were more active, had a lower swimming ability and were easier to catch, compared to non-infected copepods

(Wedekind & Milinski 1996). The increase in copepod activity could be caused by the infection and potentially be a manipulative effect of the parasite to increase conspicuousness of the host. However, another study found that the likelihood of parasite ingestion and thus infection increases with increasing copepod activity (van der Veen 2003). Consequently, infected copepods are probably the more active ones already before they get infected, so that the measured increase in activity is due to initial activity differences and not due to an infection. To our knowledge, none of the studies on differences in behaviour between infected and non-infected individuals controlled for initial behavioural differences between hosts. To exclude this possibility, we monitored activity of copepods, the first intermediate host of *S. solidus* 2 days pre infection as a self-reference.

Assuming that the monitored activity does not differ between infected and uninfected copepods prior to infection but that a difference occurs during an infection with the parasite, the timing of the behavioural change is important to understand what causes it. We therefore followed exposed and control copepods during the course of an infection with *S. solidus* and monitored activity of the same copepods several times.

To be adaptive, a behavioural trait should only change when the parasite is infective and ready for transmission to the next host to lead to an increase in parasite fitness (Poulin 1995). If such a manipulation by the parasite occurs in our system, we expect copepods to increase in activity shortly after the procercoids became infective for the three-spined stickleback. In our culture conditions this is the case around day 5-6 (pers. observation K. Hammerschmidt), the time shortly after the procercoids of *S. solidus* developed their cercomer (Clarke 1954; Orr & Hopkins 1969).

A behavioural change in infected copepods could be also a by-product of infection, caused by reallocation of resources to other life-history parameters of the copepod. We expected copepods to fight the parasite and thus increase resource allocation towards their immune system. Previous studies on this system showed that the only time, copepods can fight the parasite, is the time around day 2 post exposure, when the parasite just penetrated through the host's gut wall (van der Veen 2003; van der Veen & Kurtz, 2002). With less energy for activity, we thus anticipated all exposed copepod to decrease in activity at the first measurement post exposure to parasites.

During the course of an infection with *S. solidus*, we thus expected all exposed copepods to decrease in activity on day 2 post infection caused by reallocation of resources to the copepod immune system. Subsequently, starting at day 5 post infection, when they potentially get manipulated by the parasite, infected copepods should increase in activity.

Materials and Methods

Source of copepods and parasites

Macrocyclops albidus copepods were kept in the laboratory as described by van der Veen & Kurtz (2002). The culture originated from 80 individuals from the Kremper Au, a small river that is connected to the brackish 'Binnenwasser' near Neustadt, the source of the parasite population.

Schistocephalus solidus eggs were obtained from the mating of one pair of parasites dissected from sticklebacks caught in the 'Binnenwasser' in autumn 2003. For hatching, eggs were transferred to 20°C for 18 days and then exposed to light the day before infection to induce hatching of the eggs (Dubinina 1966).

Design of experiment

Six days before the exposure to a parasite larva, 240 adult copepod males were filtered from the culture tanks and measured. To estimate copepod size, three pictures were taken from each copepod. Repeatability of these measurements was 91% (calculated using variance components) and the mean of the three measurements was used (van der Veen 2003). After measuring, the copepods were transferred into individual wells of 24-well plates with 2 ml of water. The experiment was conducted on two groups of 120 copepods. The two groups were treated the same but processed on two subsequent days and the groups will subsequently be referred to as rounds in the rest of this paper.

At day zero of the experiment, 190 copepods were exposed to one parasite larva each, whereas 50 copepods served as a control (balanced between the two rounds). They were not exposed to a parasite larva but otherwise treated the same as the exposed copepods during the experiment. Treatments were randomized over

the 24-well plates and the person measuring copepod activity was blind concerning treatment.

To detect potential changes in copepod behaviour during the infection period, activity of individual copepods (referred to as 'hops' in the following) was measured repeatedly on 6 occasions (measurement days; Fig. 1). The first measurement took place on the 2nd day pre exposure and served as a self-control. The next measurement was done on the 2nd day post infection (early infection) to check for a potential behaviour change due to an activation of the immune system of the copepod. On day 5 post infection, the day of the next measurement, most parasites start to develop the cercomer (pers. observation K. Hammerschmidt), which is the first step of becoming infective for the next intermediate host, the three-spined stickleback (Clarke 1954; Orr & Hopkins 1969). The next measurements on day 7, 9 and 11 were done to check for changes in behaviour later during the infection process.

Two weeks post infection, copepods were screened for parasite larvae under a microscope.

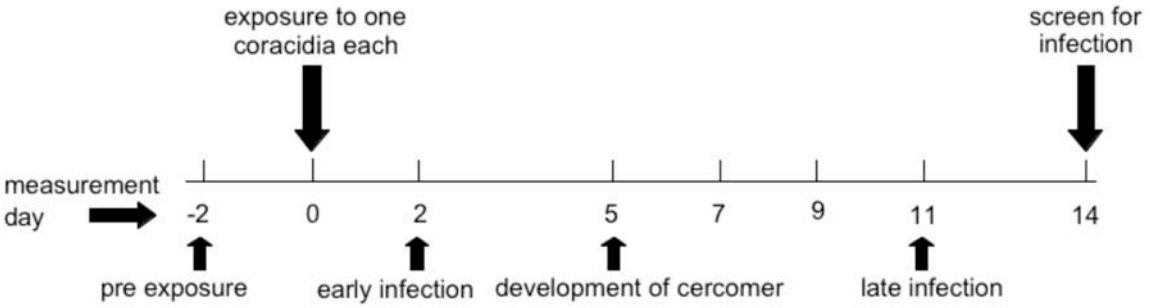


Figure I-1: Design of the experiment. The 6 measurement days are shown to monitor the course of infection: -2: self-control, 2: immune activation, 5-7: development of cercomer (getting infective for the next host), 9-11: late infection. At day 0, exposure to parasite and at day 14, screening for infection took place.

Measurement of copepod activity

To measure copepod activity, plates were put under the light of a dissection microscope for 15 to 20 minutes to customize the animals to light. Generally, light intensity was very low to minimize possible impact to the copepods (pers. observation I. van der Veen). After acclimatisation, each copepod in the plate was observed for one minute to record the hops of every copepod, as described by van der Veen (2003). The recording of hops for one minute was repeated three times on each measurement day for each copepod (with around three hours difference between measurement for each copepod) to be able to exclude 'irregular' behaviour of one copepod and minimize variance (repeatability of the activity per day by calculating variance components -2 days: 52.1%; 2 days: 39.1%; 5 days: 43.3%; 7 days: 48.3%; 9 days: 49.9%; 11 days: 56.1%). Repeatability values are quite high for behavioural traits so the mean of the three measurements was used in all analyses.

Feeding and keeping of copepods during the experiment

On the day of isolation and one day before each activity measurement, copepods were fed with 5 freshly hatched nauplii of *Artemia salina* to exclude a change in activity due to hunger or lack of energy. The only exception in feeding took place before the first measurement at two days before infection, where copepods were fed with two nauplii only to increase the likelihood of consumption and thus infection with the parasite. To reduce death rate of copepods, water was changed weekly but directly after a measurement to exclude changes in behaviour due to fresh water.

Data analysis

At the end of the experiment, we noted which copepods had died to check whether mortality of copepods depended on parasite exposure or varied between the two rounds. Accordingly, we performed two log linear models with copepod death as response and either parasite exposure (control or exposed) or round (1 or 2) as effect variables.

To test whether copepod activity changed in the course of the experiment and if this time trajectory differed between infected and uninfected copepods, we performed a repeated measurements MANOVA with time (day –2, 2, 5, 7, 9, 11) as the repeated factor (within subject), treatment (control, exposed but not infected,

infected) and round (1 or 2) as between subject class variables and copepod size as between subject continuous variable.

To detect the point in time in the trajectory where the difference in activity appeared, post hoc ANOVAs with the change in activity from before exposure (day -2) to the other days as response variable, and with treatment as effect variable were done. On day 2 we also took parasite exposure in as effect variable, because on that day, we expected a change in activity due to an activation of the immune system.

One infected copepod had to be excluded from the analysis since it was accidentally infected with two parasite larvae. In general, all insignificant interactions were removed from the models. All test statistics refer to two-tailed tests. Effects were considered significant at a level of $P < 0.05$. Analyses were performed with the JMP Version 5.0.1.2 (SAS™) software for Macintosh™.

Results

Survival and infection rate

In total, 156 copepods survived the experiment and were included in the analysis. Mortality of copepods (34.7%) did not depend on exposure to parasite (Likelihood Ratio (LR) $\chi^2 = 0.64$, d.f. = 1, $P = 0.43$) but varied between rounds ((LR) $\chi^2 = 5.58$, d.f. = 1, $P = 0.02$). Infection rate (44.6%) was not different between the two rounds ((LR) $\chi^2 = 0.02$, d.f. = 1, $P = 0.90$).

Effect of parasite infection on activity of copepods

The repeated measurement analysis revealed a significant effect of treatment across the different measurement days ($\lambda = 0.81$, $F_{10,294} = 3.20$, $P < 0.0006$). This matched the expectation that the trajectory of activity over time differed between the treatments (Table 1). In the same model, treatment was found to have a significant overall effect on copepod activity ($F_{2,151} = 6.16$, $P < 0.0027$), whereas copepod size ($F_{1,151} = 0.04$, $P = 0.84$) or round ($F_{1,151} = 0.10$, $P = 0.75$) were not significantly related to activity.

Table I-1: Mean activity of copepods (hops/min) for the three treatments for each measurement day during the course of infection with *S. solidus*.

day	copepod activity (hops/min)		
	infected (n=54)	non-infected (n=67)	control (n=35)
-2	34.07	30.20	26.99
2	28.62	26.30	29.87
5	18.60	25.79	28.55
7	13.69	21.78	26.71
9	10.52	21.54	24.69
11	13.36	22.68	26.82

With an ANOVA, it was examined post hoc, if copepods already differed in activity before exposure to the parasite, which was not significantly so ($F_{2,153} = 1.64$, $P = 0.20$). It was also examined post hoc with an ANOVA, if exposed and non-exposed copepods differed at day 2 post exposure in their activity change due to a potential activation of their immune systems, which was significantly so between ($F_{1,154} = 4.35$, $P < 0.0387$; Fig. 2). Within the exposed group, no difference in activity change could be determined between the exposed and infected copepods ($F_{1,119} = 0.20$, $P = 0.64$). From five days post infection onwards to the end of the experiment, significant changes in copepod activity due to treatment were found (5 days: $F_{2,153} = 7.46$, $P < 0.0008$; 7 days: $F_{2,153} = 8.73$, $P < 0.0003$; 9 days: $F_{2,153} = 11.78$, $P < 0.0001$; 11 days: $F_{2,153} = 9.24$, $P < 0.0002$). When comparing the least square means with a Tukey-Kramer HSD test where the ANOVA's were significant, the infected copepods were always found to be less active compared to the exposed (but not-infected) and the control ones. The repeated measurement analyses further showed a significant effect of round across the different measurement days ($F_{5,147} = 3.20$, $P < 0.03$), which was not the case for copepod size ($F_{5,147} = 1.00$, $P = 0.42$).

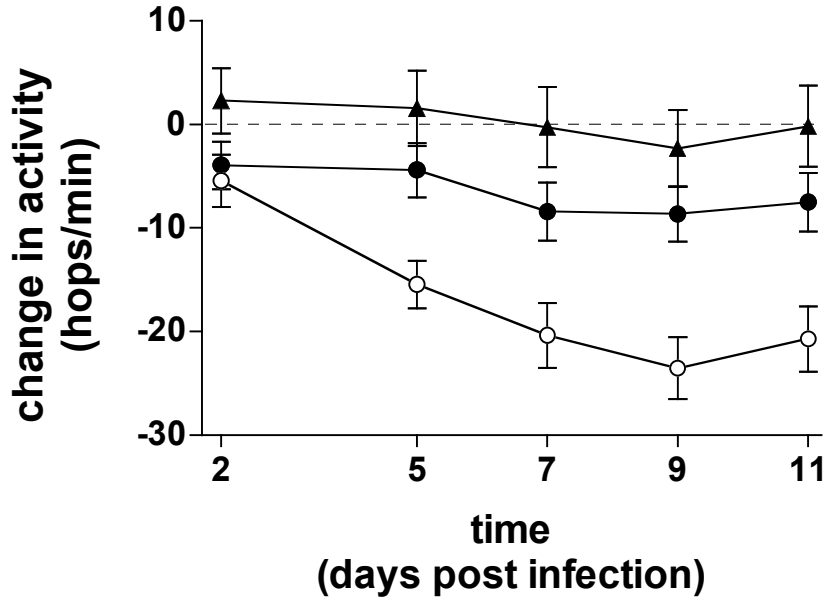


Figure I-2: Change in activity (activity of each copepod on each measurement day minus activity pre exposure; ± SE) measured as hops per minute during the course of the experiment of control (▲) (n=35), exposed (●) (but uninfected) (n=67) and infected (○) copepods (n=54).

Discussion

When comparing infected with non-infected (exposed, but non-infected and control) copepods during the course of an infection with the tapeworm *S. solidus* we found, contrary to our expectation, infected copepods to significantly decrease and not to increase in activity at the 5th day post exposure. Before exposure to parasites, copepods did not differ in activity between the three treatments. Two days post exposure, exposed copepods were less active than the control, which could be caused by a reallocation of resources from movement to the upregulation of the immune system.

For the first time, we show a change in behaviour of infected copepods in the course of infection with *S. solidus*. Compared to other studies (Urdal et al. 1995; Wedekind & Milinski 1996), where infected copepods were compared with control and exposed ones at only one occasion during infection, we followed each individual copepod through the progressing infection but also took measurements before exposure to the parasite. Doing so, we were able to exclude initial behavioural differences between copepods. We could clearly show that infected, uninfected and

control copepods did not differ in activity before they were assigned to the different treatments.

The activity change in the course of infection shows a characteristic pattern for each of the three treatments. Copepods assigned to the control group did not significantly change but kept the same activity level as 2 days pre exposure, which suggests that our experimental set up did not influence copepod activity. As expected, all exposed copepods decreased in activity, and thus significantly differed from the control group already on the 2nd day post exposure. The decrease in copepod activity shortly after exposure to the parasite could be due to a reallocation of resources to the immune system to fight the parasite. Interestingly, the exposed but not-infected copepods stayed around that activity level and showed that lowered activity compared to the control group for the rest of the experiment. Thus the activation of the immune system seems to bear costs for the copepods. However in this experiment, we cannot say if this is due to a single activation or if the immune system stays activated in anticipation of further parasite encounters.

Contrary to our expectations, copepods in the infected group further dropped in activity in contrast to the control and exposed treatments from day 5 post exposure onwards. How can such a result be explained? If the decrease in activity on day 2 post exposure was due to an activation of the immune system, which was the same for all exposed copepods, any further activity decrease in the infected group can be either interpreted as a physiological side effect due to energy drain or to a direct manipulation by the parasite.

The timing of the decrease in activity suggests direct manipulation, because the biggest change in activity happened around day 5, where procercoids of *S. solidus* start to develop the cercomer (this species under conditions used in this study, pers. observation K. Hammerschmidt), which is the first step of becoming infective for the next intermediate host, the three-spined stickleback (Clarke 1954; Orr & Hopkins 1969). Further support for direct manipulation, comes from another study in the same system, where for the time of cercomer development, an increase in carotenoid volume and thus conspicuousness was recorded (van der Veen & Hammerschmidt in preparation). One precondition for a change in behaviour to be a manipulation by a parasite is that it has to be beneficial for the parasite. Does a decrease in copepod activity increase the likelihood of *S. solidus* infected copepods

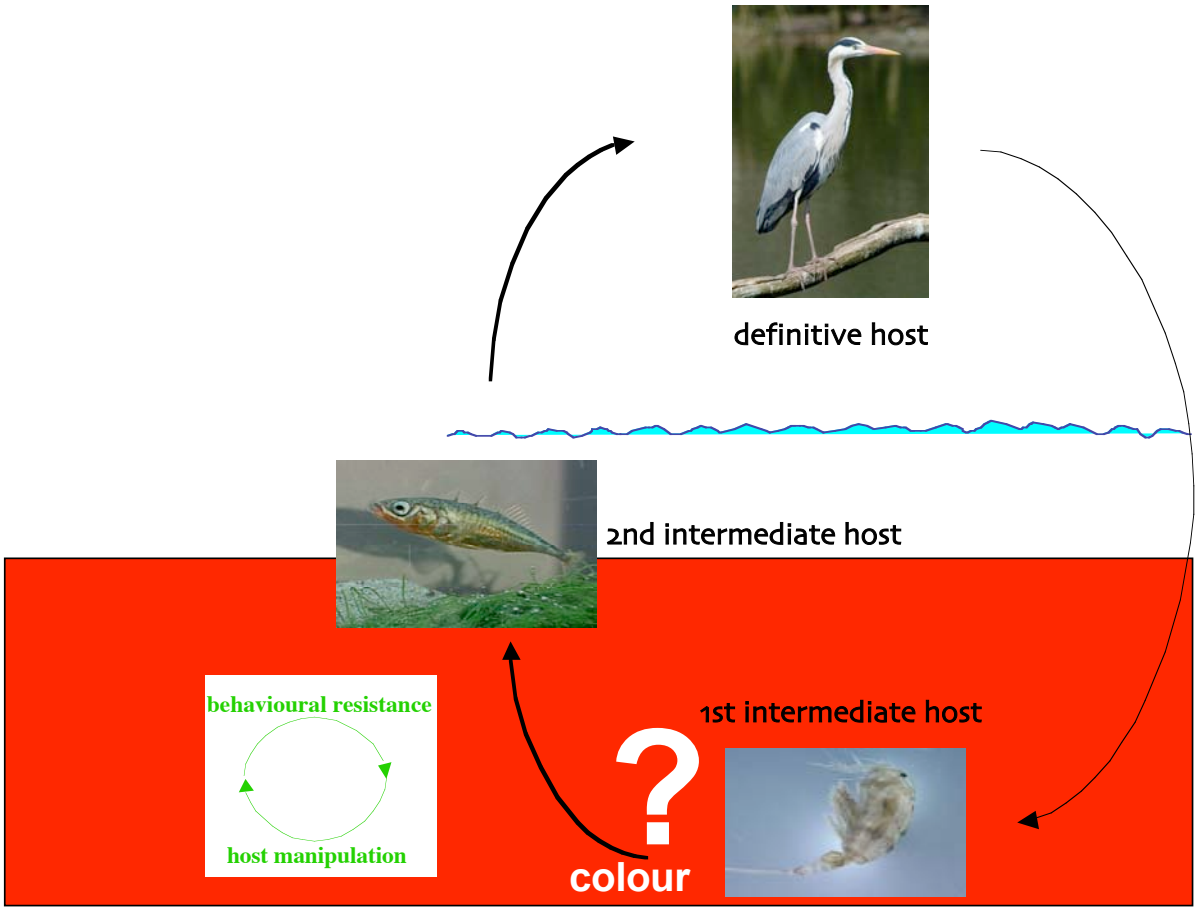
to get preyed upon by a three-spined stickleback? Optimal foraging theory predicts that predators should choose their prey in a way to maximise their net energy gain. Therefore they should prefer prey that returns the highest amount of energy when the costs for searching, pursuing and handling are also taken into account (MacArthur & Pianka 1966; Stephens & Krebs 1986; Werner & Hall 1974). When threatened by a predator, such as a stickleback, copepods try to escape with relatively big 'escape' hops (Buskey et al. 2002; Viitasalo et al. 1998). Infected copepods that have less energy for activity or 'escape hops' become easier to handle and thus a more attractive prey with a higher net energy gain for the fish (Wedekind & Milinski 1996). In a previous study infected copepods were indeed shown to be more preyed upon and easier to catch than non-infected ones (Wedekind & Milinski 1996).

The decrease in activity of infected copepods can alternatively be interpreted as a result of the physiological damage caused by the parasite, as shown in previous studies for other systems (Poulin 1995). Resources in the copepod are likely to become limited during the course of infection, because individuals of *S. solidus* and helminths in general can reach relatively large sizes in their copepod host (Clarke 1954; Guttowa 1961; Wedekind 1997; Wedekind et al. 2000). Support for the energy depletion hypothesis comes from studies on the same system, where Wedekind and Milinski (1996) found infected copepods to be less successful than uninfected ones, when swimming against a water current. In another study, it was shown, that infected copepods had higher demands on the uptake of resources, i.e. an increased hunger level than their uninfected conspecifics, (Jakobsen & Wedekind 1998). Therefore, infected copepods seem to have fewer resources available for activity than uninfected ones. The higher energy demand of infected copepods can potentially explain the results from other studies that found *S. solidus* infected copepods to be more active than non-infected ones (Urdal et al. 1995; Wedekind & Milinski 1996). Instead of parasite manipulation, the increased activity was probably due to hunger: copepods are normally 'sit-and-wait' predators that switch to an active search strategy when hunger level increases (Formanowicz 1982; Inoue & Matsura 1983). In contrast to both other studies that found an increase in activity, we tried to minimize hunger induced behavioural changes in our experiment: we fed copepods a day before activity measurements and did not transfer them to a new environment. Thus

they did not have to switch to an active hunting strategy, that normally includes an increased exploration of the environment.

In conclusion, we found no difference in copepod activity before exposure to parasites. Exposed copepods decreased in activity already at the 2nd day post exposure, suggesting resource reallocation to their immune systems. Activity declined even further in the infected group around day 5 and levelled off until the end of the experiment. In this experiment, we cannot distinguish if that is caused by energy uptake or manipulation by the parasite. The contradicting findings compared to other studies on the same system, but also the increase in carotenoid volume at the same time when we detected the activity increase (van der Veen & Hammerschmidt in preparation), indicate that interactions of *S. solidus* with its first intermediate host are complex. This fits to some recent publications, where it is suggested to understand and analyse parasitized hosts as deeply modified organisms with several modifications, some of which may favour parasites and some, which may favour hosts (Cézilly & Perrot-Minnot 2005; Thomas et al. 2005; Webster 2005).

CHAPTER II


definitive host
2nd intermediate host
behavioural resistance
host manipulation
1st intermediate host
?
colour

Colourful copepods: do parasites manipulate carotenoids?

Abstract

Complex life cycles are common among parasites. Parasites with such complex life cycles can only gain any fitness when they are transmitted to the right host at the right moment. Increasing the chances of transmission to the next host would have large benefits for hosts with complex life cycles. Although increased transmission rates through changes in host behaviour have been observed in many parasites, it is scientifically not easy to distinguish between active manipulation by the parasite or side-effects of the infection being the cause of changes in infected hosts. In this paper we showed that *Macrocyclops albidus* copepods had higher survival and stored more carotenoids when they were infected with the cestode *Schistocephalus solidus*. Larger carotenoid reserves renders copepods more conspicuous, which would lead to higher predation rates by visually hunting predators that are attracted by orange colours, like the three-spined stickleback, the next intermediate host of *S. solidus*. The change in conspicuousness appeared around the time of formation of the cercomer, which happens when the parasite becomes infective to the next host. Increased survival and conspicuousness after becoming infective both leads to higher transmission rates and thus higher fitness for the parasite. However, there seems to be an arms race going on between the parasite and the host over the conspicuousness of the host. Parasites with a larger cercomer seem to have a larger influence on the conspicuousness of the host, but large and intermediate hosts seem to be better at avoiding becoming more conspicuous. To what extent one could call the changes in infected copepods in this study manipulation by the parasite is discussed in the light of the argument put forward the literature.

Introduction

Many parasite species depend on several host species for completion of their life cycle. From an evolutionary point of view it is often not easy to explain such complex life cycles, because the evolution of complex life cycles involves many, in some cases not very likely, steps (Parker et al. 2003a; Poulin & Cribb 2002). Yet many parasites have evolved such complex life cycles. One difficult to explain aspect of complex life cycles is that in order to gain any fitness a parasite must be transmitted from one host to the next. To increase the chances of doing so, one could imagine that the parasite could manipulate the current host in a way that enhances the transmission rate. There are many examples of such proposed parasite manipulation, which include manipulation of microhabitat choice, evasive behaviour, activity, and conspicuous appearance of the host (Hurd 1990; Moore 2002), most of which will increase the predation risk of the current host causing higher transmission rates to the next host.

Even though there seem great advantages for parasites in being able to manipulate their current host to increase their chances of transmission to the next host, scientifically it remains very difficult to determine if observed changes in infected hosts are due to active manipulation of the host by the parasite or if these changes are mere side-effects of the infection that happen to be to the advantage of the parasite. (Poulin 1995) proposed several criteria for calling a change in the host a manipulation by the parasite. In order to be called a manipulation, a change has to be complex, must be purposive, has to be arisen independently in several lineages and must increase the fitness of the parasite. Even though not many studies have used these criteria or have been able to show true manipulation, the vast amount of suggestive data has made parasite manipulation a widely used term in evolutionary ecology.

Especially helminthic parasites are known to be associated with changes in their host, including changes in all traits mentioned above (Poulin 1994b). The cestode *Schistocephalus solidus*, which is the subject of the current study, also has a complex life cycle, with trophic transmission by means of predation. After hatching of the eggs in the water, the free-swimming coracidia have to be ingested by a cyclopoid copepod (Dubinina 1966). After growing considerably in the copepod body cavity, the parasite has to be transmitted to the next host, the three-spined

stickleback *Gasterosteus aculeatus*. There, again, the parasite grows considerably in the body cavity, upon which it has to be transmitted to its next host, a fish eating bird. In the gut of the bird, reproduction takes place after mating either with itself or with a partner (Smyth 1954). The bird defecates the eggs in the water, which makes the cycle complete. Looking at the complexity of this life cycle, it is clear there are several stages that would be open to manipulation by the parasite. Indeed several studies have concentrated on manipulation in this system. In the stickleback changes in host appearance (Lobue & Bell 1993), microhabitat choice (Jakobsen et al. 1988; Lobue & Bell 1993), activity (Giles 1983; Milinski 1985) and evasive behaviour (Milinski 1990) have been reported. Although these studies did not distinguish between manipulation or side-effects being the cause of the changes, studies showing that changes in evasive behaviour did not occur until the parasite was ready for transmission to the next host (Tierney et al. 1993) indicate that manipulation may play a role. (Øverli et al. 2001) proposed that changes in the brain monoaminergic activity of sticklebacks could be responsible for the observed changes. Also in the copepod host several examples of changes have been reported. In copepods the microhabitat choice (Jakobsen & Wedekind 1998), evasive behaviour (Urdal et al. 1995; Wedekind & Milinski 1996) and the activity has been shown to be altered (Wedekind & Milinski 1996; Hammerschmidt et al. in preparation). The change in activity of infected copepods has been suggested to be due to manipulation by the parasite, because the alternative explanation of energy depletion does not seem to hold (Franz & Kurtz 2002) and the timing of the change in activity coincides with the time when the parasite is ready to be optimally transmitted to the next host (Hammerschmidt et al. in preparation; Parker et al. 2003a, b).

Although changes in conspicuous appearance in intermediate hosts of trophically transmitted parasites have frequently been studied (e.g. Bethel & Holmes 1974, Bakker et al. 1997), until now there are, to our best knowledge, no studies reported on changes in conspicuous appearance in the copepod host of cestodes. It is likely to assume that in cyclopoid copepod hosts infected by cestodes alteration of conspicuousness could also be a way to gain higher predation rates to increase transmission success. Although the cestodes themselves seem colourless, copepods are known for having carotenoid based cuticle colouration (Hairston 1979a) and for storing carotenoid reserves that make them appear orange (van der Veen 2005).

Carotenoid based colouration of copepods is shown to be variable between species (Hairston 1979a) and environmental conditions (Hairston 1976, 1979a, b, 1980; Hansson 2000, 2004; Rhode et al. 2001), and carotenoid storages are shown to vary within individuals over time (Hansson 2000, 2004; van der Veen 2005). Visually hunting fish, which are often the second intermediate host in cestode cycles, have been shown to prefer to prey on conspicuously orange coloured prey (Bakker et al. 1997; Endler 1980; Hairston 1979a; Ohguchi 1981). Therefore it is likely that changes in carotenoid based colouration of copepod hosts could lead to higher fitness of cestodes through transmission rates and thus one could expect such changes to occur during infections of copepods by cestodes.

Another reason why carotenoids are interesting to study during infections is because of their role in the immune response of the host. Carotenoids are known immunostimulants (Lozano 1994), and play an important role during activation of the immune system because of their free radical scavenging capacity. During immune defence highly reactive free radicals are produced and without free radical scavengers, like carotenoids, these free radicals may damage important molecules and proteins in the body (Bendich 1989; Bendich 1993; Bendich & Olson 1989; von Schantz et al. 1999). When a host would use carotenoids for free radical scavenging purposes during an immune defence, one would expect carotenoid reserves and thus conspicuousness to decrease upon infection, which is the opposite of what is to be expected when the parasite alters the conspicuousness of its host to increase transmission success.

In studies on the orange colouration of hosts, evidence for both theories has been found. In sticklebacks infected individuals are less colourful, presumably because of worse body condition or use of carotenoids during immune defence (Milinski & Bakker 1990). Other reasons for duller colour in hosts could be that the parasite interferes with the uptakes of carotenoids by the host (Bradley et al. 2001; Ruff et al. 1974), or the parasite could be taking up carotenoids directly from the host to store them in their own body (Gaillard et al. 2004; Hindsbo 1972). However, the latter system would still lead to more conspicuous hosts as long as the host is transparent, which is for example the case in Acanthocephalan systems (Moore 2002). Some elegant studies on Acanthocephalan parasites showed that this change in conspicuousness, as well as the accompanied change in photo tactic behaviour,

leads to higher predation by the next intermediate host (Bakker et al. 1997; Bethel & Holmes 1977). In the current study we aimed to study whether changes in carotenoid storages occur in the cyclopoid copepod *Macrocyclops albidus* during infection with the cestode *S. solidus* and whether the timing of these changes coincides with the time when the parasite is ready for transmission to the next host.

Methods

Copepod maintenance

The stock population of *M. albidus* originated from the river Kremper Au, which is connected to the brackish Neustädter Binnenwasser, from which the parasite stock originated. The copepod stock was cultured as described in (van der Veen & Kurtz 2002). In the culture the copepods were kept in water in which hay had been boiled and were fed with *Paramecium caudatum*. When on a pure *P. caudatum* diet, copepods do not build up large carotenoid reserves. During the experiment copepods were kept in isolation in 2 ml of hay-water in wells of 24 well plates and were fed with 3 *Artemia salina* nauplii three times a week, starting on the day of isolation. On each feeding occasion the copepods were checked for survival. In the plates the hay-water was refreshed after each carotenoid count. The plates were kept on a 16L:8D light schedule at 20ºC.

Experimental design

To test whether infected copepods differed from uninfected copepods in their trajectory of carotenoid reserves over time we isolated 816 male copepods from the stock population. The copepods were allowed to acclimatise in the plates for 10 days before counts started. Carotenoid droplets were counted (see below) on 2 days pre exposure and on 2, 7 and 11 days post exposure to parasites (Fig 1). The 2 days pre exposure measurement was used as a self reference and day 2 was chosen as a day where the parasite is still small and does not have a cercomer yet, at day 7 all copepods are assumed to just have formed their cercomer (which takes place at day 5 or 6 under these circumstances). The development of the cercomer seems to be crucial for infection success in *S. solidus* (Clarke 1954; Orr & Hopkins 1969). At day 11 copepods are fully infective to the copepods and have grown even bigger

compared to day 7. The copepods were randomly assigned to two groups: they were either not exposed to parasites and served as controls (n=60), or they were exposed to a parasite (n=756) and ended up being either infected or uninfected. The three groups: control, exposed (but uninfected) and infected are referred to as 3 treatments in the rest of the paper. The experiment was performed in two equal rounds, where round 2 was started 3 days after round 1.

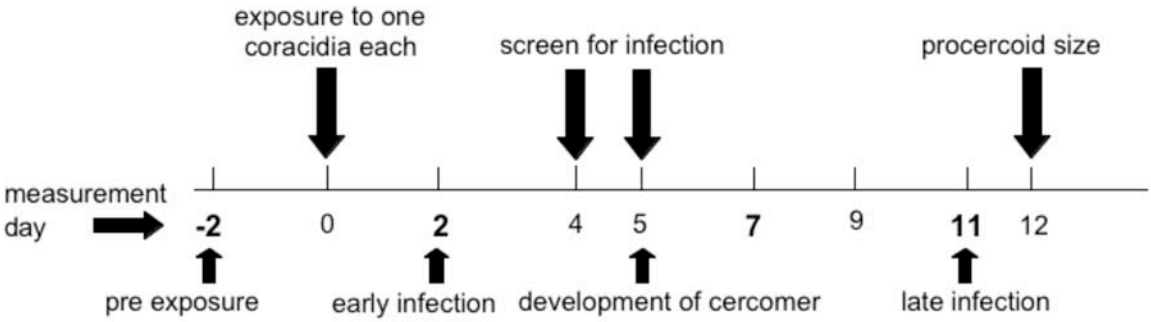


Figure II-1: Schematic figure of experimental design

Infections

Twelve days after isolation the copepods were exposed to one parasite larva each. The parasites originated from 7 clutches obtained from breeding pairs of adult parasites. The adult parasites had been dissected from wild caught sticklebacks. The parasite eggs were stored at 4°C in the dark for 37 weeks until three weeks before infection, when they were moved to 18°C. To induce hatching, the eggs were exposed to light one day before infection (Dubinina 1966). Copepods exposed as controls and to the 7 clutches of parasites were randomly divided over the plates. Although a balanced design was planned, the balance had to be broken due to low hatching rates in some of the clutches. These copepods were then exposed to a parasite from another clutch (clutch A=113, B=66, C=110, D=110, E=139, F=194, G=55 and H=79 copepods exposed). On day 4 (round 1) or day 5 (round 2) of the experiment infection status was determined of all copepods with help of a microscope. On day 12 of the experiment the parasites were removed from the copepods and fixed by adding a drop of 20% formalin to the water. Of all parasites a picture was taken with a video camera.

Size measurements

Of all copepods body size was measured as the distance between the central posterior side of the eye and the dorsal anterior end of the cephalothorax by means of image analysis (Scion Image, a modification of NIH Image http://rsb.info.nih.gov/nih-image). From each copepod three size measurements were taken (repeatability = 96.2%) and for analyses the average was used. These size measurements were done during the acclimatisation period. The pictures of the parasites were also analysed by means of image analyses; we measured the area of the body and the area of the cercomer (for details see Hammerschmidt & Kurtz 2005a; chapter V).

Counting of carotenoids

After feeding the copepods with *Artemia salina* nauplii, orange coloured lipid droplets appeared in the haemocoel. We counted these carotenoid droplets in four size categories: large, medium, small and extra small, as described in (van der Veen 2005). The order in which the plates were processed was the same on each counting day to avoid time effects causing noise in the data. Throughout the experiment the observer was blind concerning treatment. Copepods were always fed 1 day prior to counting of the carotenoids (c.f. Franz & Kurtz 2002). After counting of the droplets the carotenoid volume was estimated with help of the average droplet volume of each size class (van der Veen 2005).

Statistical analysis

The infection rate was 24 % in this experiment. Even though mortality did not differ between the exposed and the control treatment ($\chi_2^2 = 0.93$, $P = 0.63$), due to high general mortality in this experiment, only 11 control, 89 exposed (but uninfected) and 32 infected copepods survived until the end of the experiment (day 11). Since statistical power is very low for the control treatment, we decided to restrict the analyses to the exposed and the infected copepods. It is worthwhile to note that the trajectory of carotenoid reserves over time did not differ significantly between control and exposed copepods (repeated measurements MANOVA: time*treatment: wilks'λ = 0.99, $F_{3,94} = 0.23$, $P = 0.88$).

Carotenoid reserves were $x^{-1/4}$ transformed before being analysed with repeated measurements MANOVAs with time (day –2, 2, 7 and 11) as the repeated factor (within subject), treatment (exposed and infected) and round (1 and 2) as between subject class variables and copepod size as a between subject continuous variable. To establish where in time the difference in the trajectory appeared, the change from before (day –2) to the other days were post-hoc analysed with ANOVAs in which treatment (exposed and infected) and round (1 and 2) were included as between subject class variables and copepod size was included as a between subject continuous variable.

To determine whether the strength of the change from before (day –2) to late infection (day 11) depended on host or parasite characteristics we performed an ANOVA on the data of infected copepods. In this analysis we included round (1 and 2) as a class variable and host (copepod) body size, parasite body size and parasite cercomer size as continuous variables. To visualise the significant interaction between host body size and parasite cercomer size in this analysis, we equally divided the hosts into three groups (small, medium, and large) after ranking them for their body size.

To analyse survival only copepods for which the infection status was known were used, i.e. only those still alive after day 5 were included in the analysis. Those copepods were classed into three groups for survival: short: copepods that died before day 7, medium: copepods that died between day 7 and 11 and long: those still alive after 11 days. These survival classes were analysed with a linear logistic model (using the procedure CATMOD in SAS (SAS 1989)) including copepod body size as a continuous variable and treatment (exposed and infected) and round (1 and 2) as class variables.

A significance level of 0.05 was used throughout the paper. Interactions with a significance level above 0.10 were removed from the analysis. Insignificant interactions are not mentioned in the text.

Results

Carotenoids

In this experiment the time trajectory of carotenoid reserves differed between exposed and infected copepods (Fig 2, repeated measurements MANOVA: time*treatment: wilks'□ = 0.93, $F_{3,111}$ = 2.63, *P* = 0.05). An increase in carotenoids can be expected if the parasites benefit from a higher transmission because of a more conspicuous host. To test where in time this effect appears, the change from before (day –2) to each of the other days was compared between exposed and infected copepods. At early infection (day 2) the change in carotenoid reserves did not differ significantly between exposed and infected copepods (ANOVA: treatment: $F_{1,120}$ = 0.10, *P* = 0.76). Just after cercomer formation (day 7) the change in carotenoid reserves differed significantly between exposed and infected copepods (ANOVA: treatment: $F_{1,113}$ = 4.42, *P* = 0.04), such a tendency was still visible at late infection (day 11: ANOVA: treatment: $F_{1,116}$ = 3.21, *P* = 0.08). This shows that around the time of formation of the cercomer, when the parasite is ready for transmission to the next host, infected copepods become more colourful than exposed copepods.

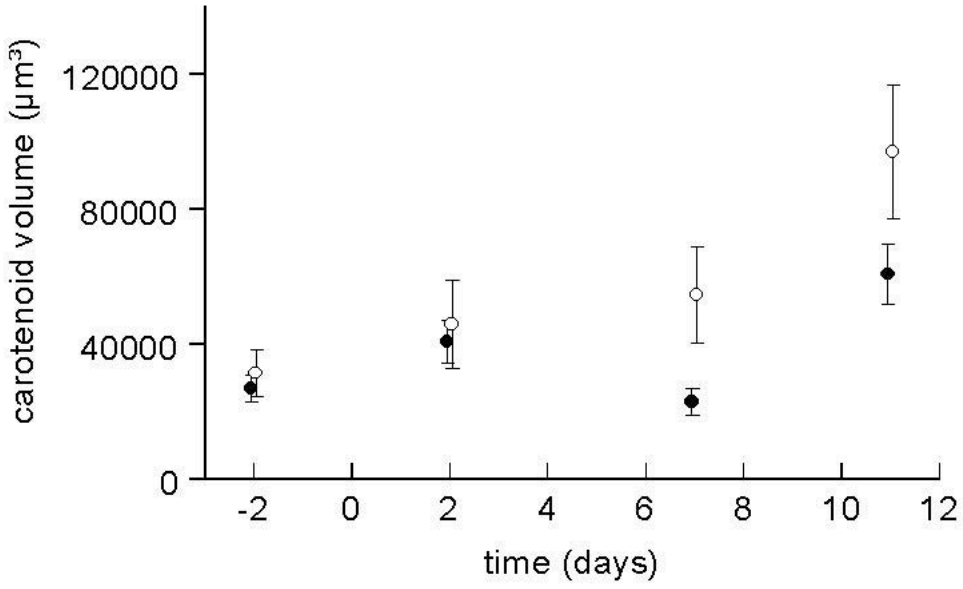


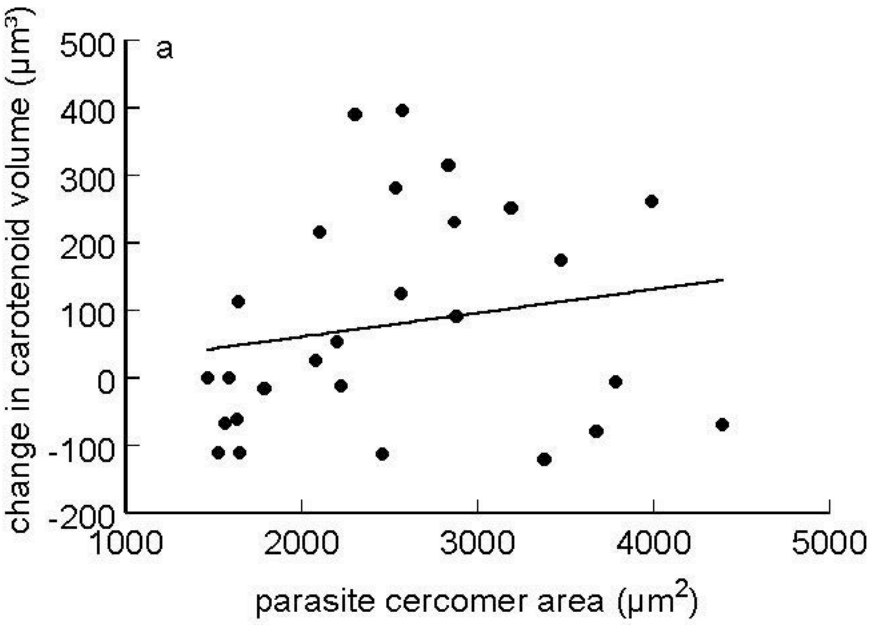
Figure II-2: Mean carotenoid volume of exposed (closed circle) and infected (open circle) copepods in the course of the experiment. Day 0 indicates the day of exposure to the parasites and bars indicate standard errors.

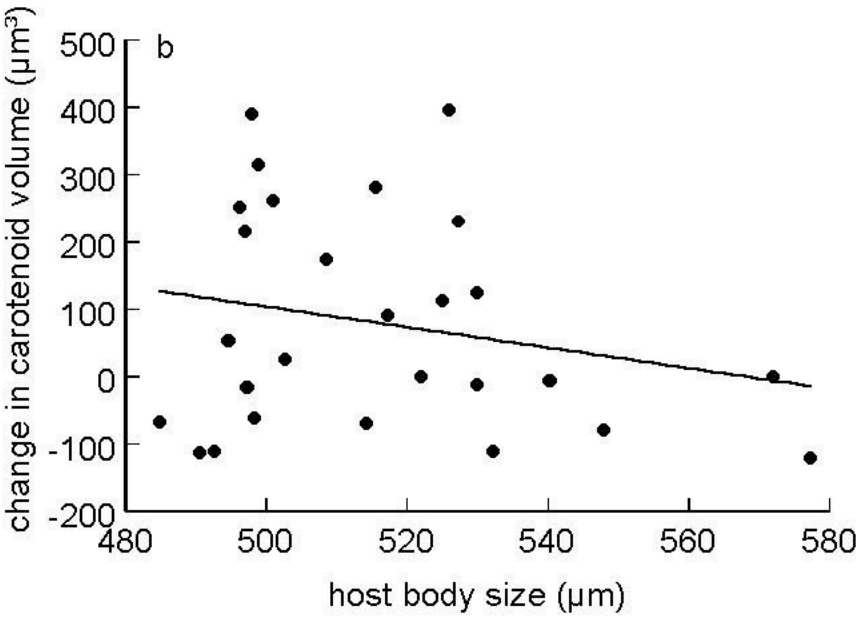
Carotenoid reserves were not significantly related to the main effects of the between-subject factors treatment, round and copepod size (repeated measurements MANOVA: treatment: $F_{1,113} = 1.21$, $P = 0.27$; round: $F_{1,113} = 0.65$, $P = 0.42$; copepod size: $F_{1,113} = 0.36$, $P = 0.55$), but there was a tendency for an overall time effect on carotenoid reserves (repeated measurements MANOVA: time: wilks'$\Lambda = 0.94$, $F_{3,111} = 2.40$, $P = 0.07$). There was a significant interaction between time and treatment and copepod size (repeated measurements MANOVA: wilks'$\Lambda = 0.93$, $F_{3,111} = 2.69$, $P = 0.05$).

Host parasite interactions

When strong colouration of infected copepods is due to parasites altering their host, one would expect characteristics of the parasite to be related to the change in colouration. On the other hand, as this is a very costly thing for the host, one may also expect some kind of counteracting force to have evolved in the host, which would be indicated by host characteristics being related to the change in colouration. Both these things seem to play a role in this study. The change in carotenoid reserves from before (day –2) to late (day 11) infection was significantly related to the cercomer size of the parasite (Fig 3a, ANOVA: $F_{1,21} = 6.83$, $P = 0.02$) and tended to be related to the body size of the host (Fig 3b, ANOVA: $F_{1,21} = 3.97$, $P = 0.06$). Moreover, the effect of parasite cercomer size interacted with the effect of host body size on the change in carotenoid reserves (ANOVA: parasite cercomer size*host body size: $F_{1,21} = 6.64$, $P = 0.02$). To visualise this latter effect we divided the hosts in three groups for body size and looked at how parasite cercomer size related to change in carotenoid reserves in each of these three groups. This shows that in small hosts there was a positive relation between parasite cercomer size and change in carotenoid reserves, whereas in medium and large hosts this effect was absent (Fig 3c).

Parasite body size was not significantly related to change in carotenoids (ANOVA: $F_{1,21} = 2.46$, $P = 0.13$), neither did round significantly affect change in carotenoids (ANOVA: $F_{1,21} = 3.77$, $P = 0.07$).


a
change in carotenoid volume (µm³)
500
400
300
200
100
0
-100
-200
1000
2000
3000
4000
5000
parasite cercomer area (µm²)


b
change in carotenoid volume (µm³)
500
400
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0
-100
-200
480
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520
540
560
580
host body size (µm)

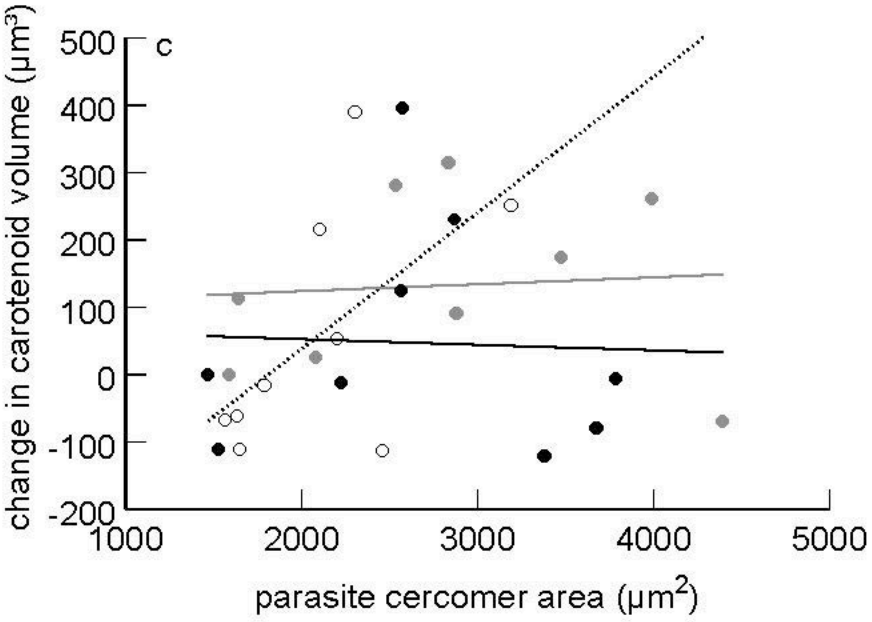


Figure II-3: Change in carotenoid volume from before (day –2) to late infection (day 11) in infected copepods related to parasite cercomer volume (a), host body size (b) and parasite cercomer volume (c) for three size classes of copepods: small (open circles, dashed line), medium (grey circles, grey line), and large (black circles and black line). Lines are depicted to visualise the effects, not to indicate significance.

Survival

Parasites gain fitness advantages when their host survives until they are ready for transmission to the next host. Therefore, one may expect survival to differ between treatments. Survival, analysed as short, medium, or long (see methods), differed significantly between exposed and infected copepods (linear logistic model: χ_2^2 = 6.77, P = 0.03). Among the infected copepods, relatively more copepods had long survival (Fig 4). The rounds also differed in their survival (linear logistic model: χ_2^2 = 22.81, P = 0.00). There was no significant effect of copepod body size on survival (linear logistic model: χ_2^2 = 2.25, P = 0.32).

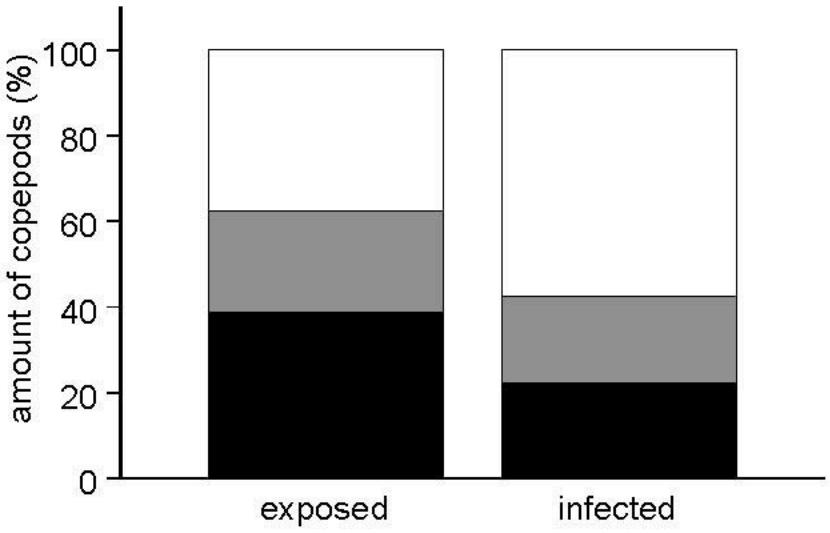


Figure II-4: Survival of copepods depicted as percentage of exposed or infected copepods that survived short (5-7 days, black), medium (8-11 days, grey) or long (>11 days, white) time.

Discussion

This study shows that infected copepods survived longer and stored more carotenoids. The increase in carotenoid reserves made the infected copepods more conspicuousness and this happened at the time when the parasite was ready for transmission to the next host. In a system where the parasite is dependent on predation of the current host by the next host, an increase in survival until the time of transmission and an increase in conspicuousness of the current host at the time when the parasite is ready for transmission will have large fitness advantages for the parasite.

In general hosts and parasites have opposite interests and therefore, and evolutionary arms race between the host and the parasite may arise. Such arms races have been studied, mainly focusing on gene frequencies or virulence (Ebert & Hamilton 1996; Lively & Dybdahl 2000; Ridley 1993). However, none of these studies focuses on an evolutionary conflict over the conspicuousness of the host. Although increased conspicuousness of the host is in favour of the parasite when the parasite depends on a visually hunting predator as the next intermediate host, becoming more

conspicuous is not in favour of the host. Therefore, one may expect an arms race between the host and the parasite about the conspicuousness of the host. When for the host as well as for the parasite traits that are related to individual quality are related to the outcome of conspicuousness, one may assume that an arms race is going on over the outcome of the conspicuousness of the host. For the parasite as well as for the host, body size is a good indicator of individual quality, as fitness increases with body size (Schärer et al. 2001; Wedekind 1997). In the current study we did not find evidence for an effect of parasite body size on the strength of the change in conspicuousness of infected hosts. Even so, since the parasite cercomer size interacted with the host body size when it comes to the change in carotenoid reserves of the host we propose that an arms race is going on. The role of the cercomer in influencing the conspicuousness of the host is currently not known. This study indicates that the outcome of this arms race is not purely in favour of the parasite. Although parasites with a larger cercomer seem to be better at altering their host's carotenoid reserves than small parasites, large and intermediate hosts seem to be able to resist this alteration.

Of course, the question remains if our observations indicate that *S. solidus* manipulates the conspicuousness and survival of its first intermediate host to increase its transmission success and thus its fitness, or if these observations are mere side effects of the infection, which happen to be beneficial for the parasite. (Poulin 1995) argued that for a change in behaviour in an infected host to be called adaptive, it has to satisfy certain conditions: (1) it has to be complex, (2) it must show signs of purposive design, (3) it has to be arisen independently in several lineages of hosts and parasites and (4) it must show to increase the fitness of the host or the parasite. Below we will discuss the significance of these conditions for the increase in conspicuousness in the system under study.

(1) The less complex an apparent manipulation of a host is, the more likely it is it has been arisen as a by-product of the infection (Poulin 1995). Carotenoid reserves differ between species and populations of copepods (Hairston 1979a) and differ between habitats in the sense that carotenoid reserves are low under predation risk and high under high UV exposure (Hairston 1979a; Hansson 2000, 2004). It has previously been shown that individual copepods are able to adjust their carotenoid reserves to prevailing predation risk (van der Veen 2005). As changes in carotenoids

in copepods seem very common, one may argue that the increased conspicuousness in infected hosts as found in this study could be a by-product of the infection. However, what makes the current study complex is the timing of the alteration of carotenoid reserves. The most apparent change in carotenoid reserves occurs around the time when the parasite is ready for transmission to the next host around formation of the cercomer. It could be argued that this is simply due to the increasing size of the parasite and thus to the increasing side effect caused by the infection. However, the parasite continues to grow after cercomer formation, but the difference between exposed and infected copepods does not seem to increase after cercomer formation. Moreover, parasite body size was not related to change in carotenoid reserves, whereas parasite cercomer size was. Because the trait under study was altered at the moment when it is most beneficial for the parasite, we argue that this is a complex alteration. Other studies have used this as an argument as well, including studies on changes in conspicuousness in Acanthocephalans (Bethel & Holmes 1974) and changes in behaviour of copepods infected with cestodes (Poulin et al. 1992; Pulkkinen et al. 2000; Urdal et al. 1995). Another argument of complexity of the observed effects is the finding that the strength of the change is related to the cercomer size of the parasite and to the body size of the host. This indicates that the observed change in colour may be the result of an evolutionary arms race between the host and the parasite over the colour of the infected host.

(2) In order for a change in behaviour to be adaptive, its design should be purposive. Adaptive traits should fit their purpose so well, that it is unlikely they have arisen by chance and more likely they have arisen through natural selection (Poulin 1995). When we a priori would predict a design that increases the fitness of the parasite in this system, we would predict an adaptation that increases the conspicuousness of the first intermediate host through red colouration. This is because, at this stage *S. solidus* can only complete its life cycle when it is transmitted to a three-spined stickleback and three-spined sticklebacks are visually hunting predators that are known to prefer to prey on red prey (Bakker et al. 1997; Ohguchi 1981). Therefore, when it comes to increasing transmission rates to three-spined sticklebacks specifically, increasing your host's red colouration seems to be the best option to go for and thus one could argue that it is likely that this adaptation has not arisen by chance but is shaped by natural selection.

(3) If an alteration independently has arisen in several independent lineages, the chances are high that this alteration is not a simple side effect of the infection (Poulin 1995). We did not do phylogenetic studies on this phenomenon. However, there are several examples from host parasite systems that show changes in colouration of a host when the parasite depends on visually hunting prey as the next intermediate host. The interesting aspect of these examples is that the way the conspicuousness of the host was altered, differs between the systems. In contrast to the current study, in the Acanthocephalan system the parasite alters the conspicuousness of the host by increasing its own colour instead of by increasing the colour of its host (Bakker et al. 1997; Gaillard et al. 2004; Hindsbo 1972; Moore 2002).

(4) In order for a change in a trait to be called an adaptive manipulation by a parasite, it should have clear fitness benefits for the parasite (Poulin 1995). Although we did not test this in this study, there are several studies that indicate that this should be the case. First of all, for the same system Wedekind & Milinski (1996) showed that *M. albidus* copepods infected with *S. solidus* suffered a higher predation risk than uninfected *M. albidus* copepods. Furthermore, there are several studies that show that sticklebacks, the next intermediate host, preferentially prey on red prey (Bakker et al. 1997; Ohguchi 1981). Some elegant studies, (Bakker et al. 1997; Bethel & Holmes 1977) even showed that the increased predation risk of *Gammarus* spec. infected with Acanthocephalans was due to the increased orange colour as well as to a change of activity of the infected hosts.

Even though on every aspect of the arguments Poulin (1995) put forward it seems possible to argue that the results found in this study point at manipulation of the host by the parasite, we feel that it is not reasonable to rule out the alternative explanation of side-effects causing the phenomenon observed in this study. It could, for example, be that the infected copepods stored large carotenoid reserves to be able to boost their immune system to fight off the parasite (Bendich 1989, 1993; Bendich & Olson 1989; von Schantz et al. 1999). Alternatively, since *S. solidus* is assumed to suppress the immune system of the stickleback host (Scharsack et al. 2004), it could be that this is taking place in the copepod host as well. In that case, carotenoid reserves may be building up, because the immune system is repressed and does not use the carotenoids.

Unfortunately, due to very low survival, we were forced to restrict the comparisons in this paper to the exposed but not infected and the infected copepods, leaving the control treated copepods out of the analysis. Although this was not the set-up we had in mind, it is what we had to work with and it should be pointed out here that one has to be cautious interpreting the remaining results. We are still confident that the data we presented here are very interesting, because of two reasons. Firstly, one important advantage of comparing these two groups is that all copepods were treated in exactly the same way: all were exposed to parasites and had probably gone through some form of immune defence. In one group this immune defence had been successful, whereas in the other group it had not. Secondly, what makes us confident of leaving the controls out of the analysis is that when looking at the data and comparing the control treatment with the exposed but uninfected treatment, no significant differences could be found. This indicates that the exposed but uninfected copepods responded largely in the same way as the controls, making it safer to use the uninfected copepods as controls in this experiment.

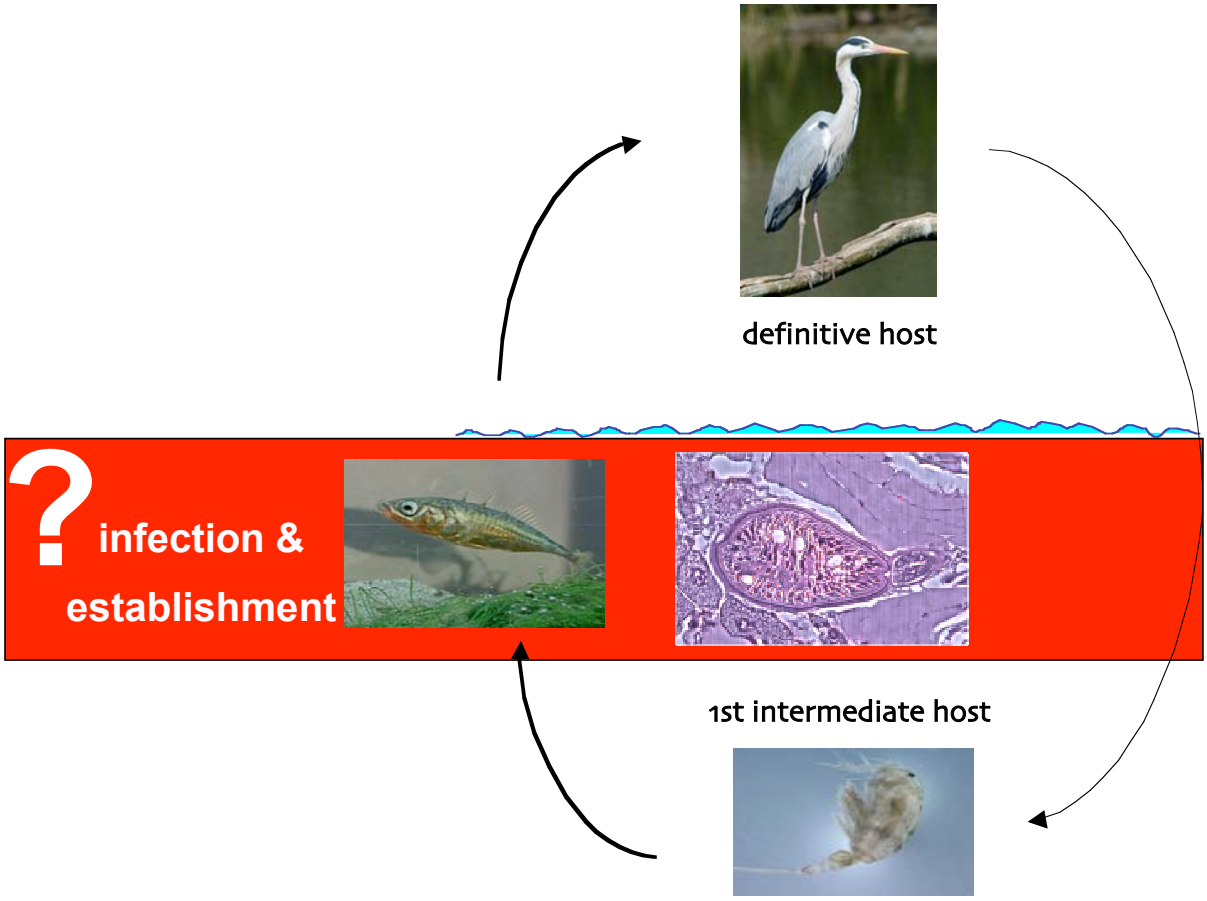
When survival of the host increases, as in the current study, survival of the parasite also increases, which, obviously, is a large fitness advantage for the parasite. In other studies on cestodes in cyclopoid copepods no effects (Pasternak et al. 1999; Wedekind 1997) or negative effects of parasite infection on survival (Nie & Kennedy 1993; Pasternak et al. 1999) have been found. However, the comparison with other studies is difficult because all these studies were done on multiple infections or looked at survival long after the parasite has become infective to the next host. In our study we only looked at singly infected copepods, which reflects the natural occurrence of infection intensity in the population where we took the parasites from (Sivars Becker & van der Veen 2004) and we looked at survival until just after the parasite became infective.

Changes in behaviour of copepods infected with cestodes have repeatedly been reported, all of which could either be due to manipulation of the parasite or by-products of the infection. Among them, deteriorated anti-predator behaviour has been reported in *M. albidus* infected with *S. solidus* (Jakobsen & Wedekind 1998; Wedekind & Milinski 1996) as well as in studies on other cestodes in cyclopoid copepods (Pasternak et al. 1995). *S. solidus* has also been found to influence microhabitat selection in cyclopoid copepods (Urdal et al. 1995) and so have other

cestodes (Pulkkinen et al. 2000). Feeding rate has also been observed to be lower in cyclopoid copepods infected with cestodes (Pasternak et al. 1995; Pasternak et al. 1999). For activity of infected copepods, different studies have found apparent contrary results. For *M. albidus* infected with *S. solidus*, activity has been found to increase upon infection (Wedekind & Milinski 1996) as well as to decrease upon infection (Hammerschmidt et al. in preparation). Similar contradictions have been found for other cestodes infecting cyclopoid copepods, where in some studies activity increased (Poulin et al. 1992; Pulkkinen et al. 2000; Urdal et al. 1995) and in others activity decreased upon infection (Pasternak et al. 1995; Pulkkinen et al. 2000). (Poulin 1995) put forward that one can argue that increased activity, as well as decreased activity can be in favour of the parasite when it comes to transmission to the next host and both could potentially be the result of parasites manipulating the host. However, to our best knowledge there are no studies on cyclopoid copepod-cestode host parasite systems that focus on changes in colour or carotenoids in cyclopoid copepods infected with cestodes. Maybe this due to the fact that in such systems behavioural manipulations may be of more importance than changes in conspicuousness (Poulin 1995).

In a study with the same set-up as the current study we found that activity was lower in infected copepods than in uninfected copepods. This decrease in activity occurred at the same moment at which in our study changes in colour started to appear, i.e. around cercomer formation (Hammerschmidt et al. in preparation). This indicates that after formation of the cercomer, parasites could benefit from increased transmission success due to increased conspicuousness as well as due to decreased activity of their current host. On top of that infected copepods had a higher survival rate than uninfected copepods, which also leads to higher fitness of the parasite. However, this study also indicates that the parasite is not always the winner in this system. Our data indicate an arms race between host and parasite, which is more in favour of the host when the host is large and more in favour of the parasite when the parasite has a large cercomer. How precisely a large cercomer may lead to a benefit for the parasite, remains an open question.

CHAPTER III


definitive host
? infection &
establishment
1st intermediate host

Fast food or fast lane: tapeworms on their way through the gastro-intestinal tract

Abstract

The penetration of the intestinal mucosal wall is thought to be a major factor determining host resistance and parasite infestation in helminth parasites. However, little is known about the time needed for infestation and selection pressures on parasites between ingestion by hosts and settling in the target tissues. The identification of components determining host resistance could be important for the development of anti-helminth drugs. We studied the establishment process of *Schistocephalus solidus* in its second intermediate host, the three-spined stickleback. As all tapeworms, individuals of that species are orally taken up by the intermediate hosts and have to leave the gastro-intestinal tract for the body cavity. In the stickleback, we found, that the worms penetrate the anterior part of the midgut within 14 to 24 hours, spending most of the time in the stomach and only a very short period in the gut. We focused on the role of different parasite surface layers and their potential function such as protection against digestion and immunity and found that the tapeworms loose an outer layer, together with the cercomer already in the gut lumen. Once exposed, the underlying tegument with microtriches seems to enable migration of the parasite into the body cavity.

Introduction

Parasites are generally supposed to have a serious impact on host fitness, which may lead to selection pressure on the host to maximize resistance and on the parasite to increase infection success. This can result in an arms race of host parasite counter adaptations so that the measured infection success of a parasite depends on the interaction between hosts and parasites (Hamilton et al. 1990; Hamilton & Zuk 1982).

For research of the evolutionary ecology of host parasite interactions, the tapeworm *Schistocephalus solidus* has become an important model organism (Christen et al. 2002; Jakobsen & Wedekind 1998; Kurtz 2003; Lüscher & Milinski 2003; Smyth 1946; Wedekind 1997). This tapeworm has to pass through two intermediate hosts, a copepod such as *Macrocyclops albidus* and the three-spined stickleback *Gasterosteus aculeatus* before it reaches its definitive host, any species of fish-eating bird. The life cycle of *S. solidus* resembles that of a typical tapeworm, with intermediate stages living in various body tissues, here the body cavity of both intermediate hosts, whereas as adults they reside in the gastrointestinal system of their definitive host, here in the gut of birds (Mulcahy et al. 2004). *S. solidus* infection leads to serious fitness costs basically in the intermediate hosts, which likely results in strong selection for defence mechanisms (Arme & Owen 1967; Kurtz & Franz 2003; van der Veen & Kurtz 2002).

How can a host increase its resistance and the parasite the probability of infection and establishment? During the infection process, parasites and hosts have several succeeding phases of attack and defence. Resistance could occur already prior to infection, such as in the case of behavioural resistance, where a host might avoid consuming infected prey, while a parasite might try to increase its intermediate hosts' attractiveness as prey (Bakker et al. 1997; Hart 1997). However, in the current host parasite system, sticklebacks seem to be unable to avoid eating copepods that are infected with *S. solidus* (Wedekind & Milinski 1996).

Little is known about the next phase of infection, the time after a parasite has entered its host but before it gets established in its final localisation within the hosts' body. Like most other helminths (Mulcahy et al. 2004), *S. solidus* infects its three hosts via the oral route. In the copepod host, the time-period between ingestion of the parasite larvae and the penetration of the copepod gut wall was shown to be of

crucial importance in determining the outcome of infection (van der Veen & Kurtz 2002). In the fish host, *S. solidus* penetrates through the mucosal barrier of the gastro-intestinal tract. However, little is as yet known of the efficiency with which the tapeworms enter the body cavity. It is also unclear so far, where and when *S. solidus* leaves the gastrointestinal tract. It is assumed, but not yet shown in a controlled experiment, that the parasite needs between 2 hours and 2 days to reach the body cavity by penetrating its host's gut wall (Clarke 1954; Dubinina 1966). Thereby it is also expected to loose its cercomer, a caudal appendix with an as yet unclear function, which is a clearly visible step of its transformation from the procercoid stage into the next developmental stage, the plerocercoid (Clarke 1954; Dubinina 1966; Tierney et al. 1993).

Once the parasite has established itself at its final destination inside the host, the only chance to eliminate the parasite seems through an efficient immune system. However, immune defence is considered to be costly for hosts due to energetic demands and potential immunopathology (Lochmiller & Deerenberg 2000; Moret & Schmid-Hempel 2000; Råberg et al. 2000; Svensson et al. 1998). Costs of highly activated innate defence mechanisms upon infection with *S. solidus* seem to harm stickleback body condition (Hammerschmidt & Kurtz 2005a; Kurtz et al. 2004; chapter V). Selection should thus favour hosts that manage to eliminate the parasite at an early stage (in the stomach or gut), ideally before it gets established at its final site within the body cavity. In turn, host counter-measures against infection might select for parasites that are able to overcome these barriers, e.g. develop a protective surface to minimize the risk of digestion. In an earlier study, we found that individuals of *S. solidus* change their surface carbohydrates upon host switch from the copepod to the stickleback. We assumed that they loose their outer layer, which could play a role in protection against the enzymatic digestion in the stomach and intestines of the fish, when penetrating into the body cavity (Hammerschmidt & Kurtz 2005b; chapter IV).

This phase, shortly before and during penetration of the intestine, is considered to be highly relevant in many parasite species. Especially in metacestode infections, the resistance against penetrating oncospheres, like *Taenia pisiformis*, is supposed to be expressed already at the intestinal level (Barker 1970; Gemmel 1964). The successful overcoming of the mucosal barrier seems important in various host

parasite interactions, including parasites of medical or veterinary importance, like ascarids and liver flukes, which have migratory stages that leave the intestine for other body tissues (Mulcahy et al. 2004).

To clarify if the gut wall really acts as a potential barrier against infection or which other factors determine host resistance, more information about the process between infection and establishment of a parasite with a migratory phase is needed. We used the tapeworm *S. solidus* in its second intermediate host to gain more knowledge about the succession and the mechanisms of the different phases of an infection. Specifically, we monitored the chronology of an infection with *S. solidus* by determining the number of parasites in the fish stomach, gut and body cavity at different points in time post exposure to the parasite. Using histological sections, we analysed where exactly and how the parasite penetrates into the body cavity of the three-spined stickleback. We also examined the different surface layers of the parasite as a link to our previous study on their protective role against host immune defence (Hammerschmidt and Kurtz 2005b; chapter IV).

Materials and methods

Infection of stickleback

Before sticklebacks (the second intermediate host) can be exposed to the tapeworm, copepods (the first intermediate host) have to be infected. For infection of copepods, instead of individual parasite exposure in previous studies, we here used a mass-exposure technique. Copepods of the species *Macrocyclops albidus* were bred in the laboratory as described by van der Veen and Kurtz (2002) and then pooled from five breeding tanks into a 5 l tank to achieve a high host density. For infection of the copepods, newly hatched coracidia (from eggs of 5 worm pairs) were added to the aquarium. After two weeks, a sub-sample of the copepods was screened as described by van der Veen and Kurtz (2002) for an estimation of infection rate, which was about 70%.

We used copepods from this infected population for infection of lab bred sticklebacks. Prior to exposure, the sticklebacks were individually transferred into small tanks with 2 l water and starved for a week to enhance consumption of copepods. Sticklebacks were then supplied with an equal amount of copepods (ca.

50 each). They started feeding on them immediately until satiation. Sticklebacks were removed from their tank at diverse time points (see below) after start of exposure and dissected immediately. This implies that although most parasites found in the fish will likely stem from the beginning of the exposure period, fish might later continue to feed on copepods. The estimated speed with which parasites enter the host body will thus be a conservative estimate.

In the first experiment, we wanted to get an estimation of the time when plerocercoids start appearing in the body cavity. We thus exposed 10 sticklebacks to infected copepods and sacrificed two sticklebacks each at 2, 6, 12 and 24 hours post exposure (p.e.). In the second experiment, we focussed on the time period that turned out most relevant in the first experiment, i.e. 12 to 24 hours p.e.. We thus dissected three fish each at 14, 16, 18, 20 and 22 hours p.e..

In both experiments, the fish were dissected and the body cavity of the fish was subsequently screened carefully for tapeworms by rinsing tissues with phosphate-buffered saline (PBS). The intestines (stomach and gut) were preserved in 4% formalin for histology.

Histological analysis

For histology, the stickleback intestines were dehydrated in a graded ethanol series and embedded in cold-polymerizing resin (Technovit 7100, Kulzer, Germany). Continuous series of sections (3µm) were cut on a Leica RM 2165 rotation microtome, placed on microscope slides, allowed to dry and stained with haematoxylin and eosin.

The sections were examined in light microscopy to determine the number of tapeworms in stomach and gut. Segments of the stickleback gastrointestinal tract were assigned as shown in Fig. 1. Since we had continuous and ordered series of sections, we could reconstruct the position of worms that might extend through up to 72 sections. For a comparison of the different stages, images were taken from parasites in the stomach, the gut, the gut wall and the body cavity with a video camera mounted on a microscope. Parasites with clear disintegration of tissues were regarded as dead (we also found parasites with intact tissues in the same gut sections, so that a preparation artefact can be excluded). When screening for parasites, we did not find any in the gut or body cavity up to 12 hours post exposure.

Therefore, we only cut and stained the sections for one fish at each, 2, 6 and 12 hours p.e..

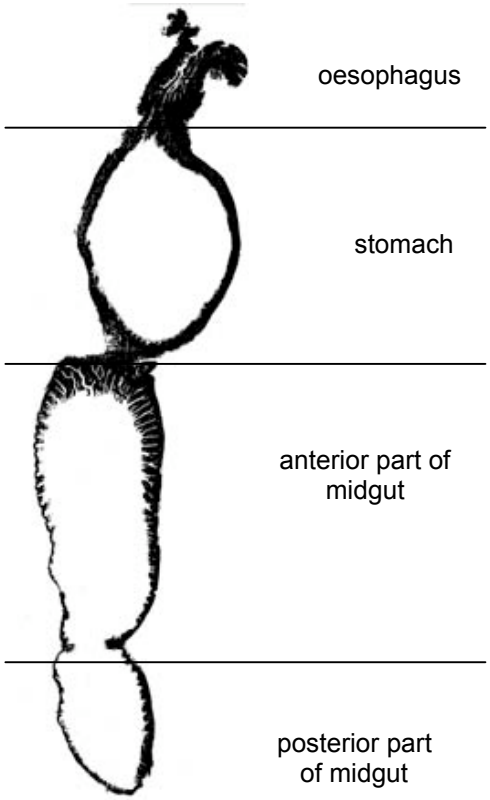


Figure III-1: Schematic overview of a longitudinal section through the gastro-intestinal tract of a stickleback.

Statistical analysis

We analysed how the proportion of tapeworms in the stomach and in the body cavity changed over time. To this end, we calculated for each fish the percentage of all worms that were found in the stomach and in the body cavity. With these values, we then did separate regression analyses for each of the two locations against time after exposure. We were also interested in how the proportion of dead to alive tapeworms in the gut changed over time. Therefore we calculated for each fish the percentage of all dead worms that were found in the gut (as measured by the total amount of parasites found in the gut) and did a regression analysis against time after exposure.

Two fish were uninfected and thus excluded form analysis. This resulted in a sample size of 18 fish for the analysis of worms in the stomach and body cavity. We did not find parasites in the gut of all fish, resulting in a sample size of 10 fish for the

analysis of worms in the gut. We considered effects significant at a level of $P < 0.05$ (two-tailed). Analyses were performed with the JMP V. 5.0.1.2 (SAS) software for Macintosh.

Results

Chronology of infestation

In the first experiment, where we dissected two sticklebacks each at 2, 6, 12 and 24 hours post exposure (p.e.), plerocercoids first appeared in the body cavity at 24 hours p.e.. We thereupon examined the histological sections of the stickleback stomach and intestine (Fig.1) for one fish each dissected 2,6 and 12 hours post exposure (p.e.), and for both sticklebacks at 24 hours p.e. These sections showed that at 2, 6 and 12 hours p.e., all parasites were still in the stomach, while none had arrived in the gut or in the gut tissue (Fig. 2; table 1). By contrast, 24 hours p.e., where we had found most plerocercoids in the body cavity of the sticklebacks, the histological sections also uncovered one parasite that was penetrating the gut tissue, as well as one worm that was still in the stomach.

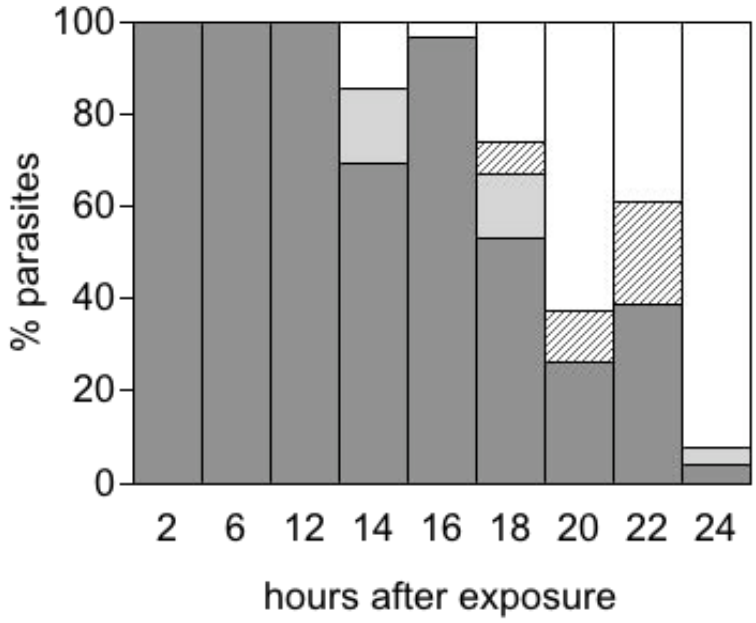


Figure III-2: *S. solidus* tapeworms in the stomach (dark grey), gut (alive = light grey, dead = hatched) and body cavity (white) in sticklebacks during a time transect after exposure, given as the proportion (%) of all tapeworms recovered per fish. See table 1 for more details.

To get a more detailed understanding of the timing of penetration, we focussed on the decisive period between 14 and 22 h p.e. in the second experiment, now dissecting three fish every two hours. We found first parasites in the stomach, the gut, the gut wall and the body cavity at 14 hours p.e. (Fig. 2; table 1). Interestingly, we did not find any intact parasites in the gut later than 18 hours p.e. in this second experiment. All parasites that were still in the gut after that time-point seemed in a digestion process, with the parasite parenchyma exposed to the host's intestinal enzymes (Fig. 4).

Table III-1: Mean number of *S. solidus* tapeworms recovered by dissection and histological analysis from individual sticklebacks at different times post exposure.

hours post exposure	Experimental round	n fish	mean n parasites (per fish)			Mean parasites (per fish)
			stomach	gut	body cavity	
2	1	1	5	0	0	5
6	1	1	28	0	0	28
12	1	1	14	0	0	14
14	2	3	6	2	1.3	9.3
16	2	3	7.6	0	0.3	7.9
18	2	3	7.3	2.3	2.3	11.9
20	2	3	1.6	0.6	3.6	5.5
22	2	3	1.3	0.6	1.3	3.2
24	1	2	0.5	0.5	8	9

Statistical analyses (pooling data from the 1st and 2nd experiment) showed that the percentage of parasites that remained in the stomach decreased significantly over time ($F_{1,16} = 20.47$, $P = 0.0003$). In the body cavity, the percentage of parasites increases significantly with time ($F_{1,16} = 14.09$, $P = 0.0017$). In the gut, we classified the found parasites either as alive or dead. There was an insignificant trend for finding relatively more dead parasites in the gut over time ($F_{1,8} = 2.57$, $P = 0.1475$).

Site of penetration

From 14 hours p.e. onwards, we not only found parasites in the stomach (Fig. 3 A) but also in the anterior part of the gut (Fig. 3 B), the gut wall (Fig. 3 C) and the body cavity (Fig. 3 D) of the sticklebacks. We never found any parasite penetrating the stomach wall, which indicates that procercoids of *S. solidus* penetrate through the wall of the anterior part of the midgut into the body cavity. All parasites, which we

observed in the gut wall (n=6) did not penetrate through the cells itself but through the relatively large interspaces between the cells in the lamina propria (Fig. 3 C).

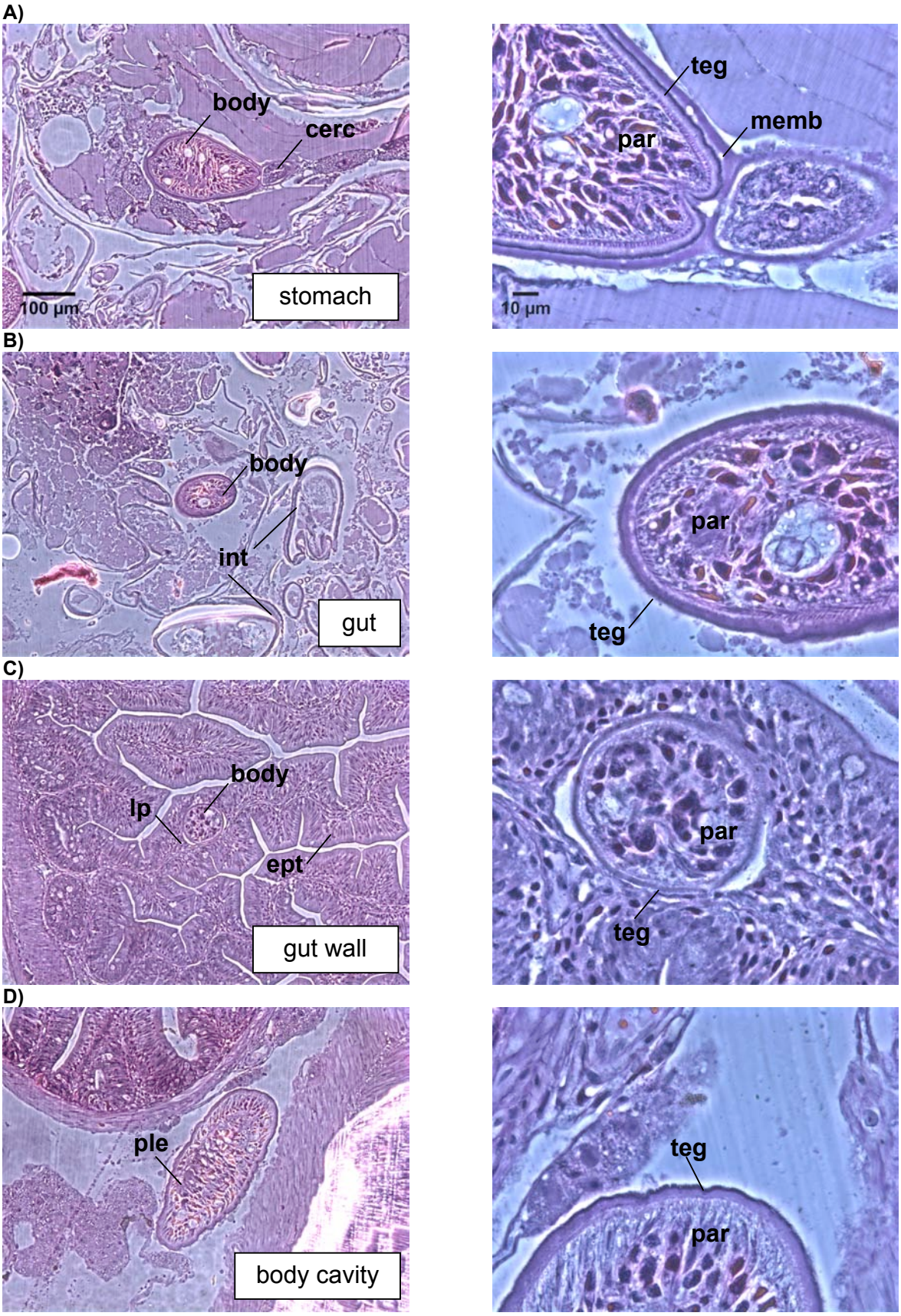


Figure III-3: Micrographs of histological sections through the gastro-intestinal tract of sticklebacks carrying *S. solidus* infections. Left: overview (phase contrast, 20x), right: detail (phase contrast, 100x). (A) Longitudinal section of a procercoid in the stomach of a

stickleback. Cercomer (cerc) and body of the parasite are clearly visible and connected by a covering thin membrane (memb), which directly covers the outer level of the tegument (teg) with its microtriches. The tegument surrounds the parenchyma (par). (B) Cross section of a plerocercoid like stage in the anterior part of the gut of a stickleback. The tegument (teg) confines the parasite from host tissue. Undigested pieces of the copepod integument (int) are also present in the gut. (C) Cross section of a plerocercoid like stage penetrating the gut wall of a stickleback in the anterior part of the intestine. After penetrating through the epithelium (ept), the parasite migrates through the interspaces between cells of the lamina propria (lp), with the tegument (teg) facing towards the gut. (D) Longitudinal section of a plerocercoid (ple) in the body cavity of the fish. The tegument (teg) faces towards the host

Surface layers

To find out when the tapeworms loose their cercomer and outer surface layers, we more closely inspected the sections of worms in the different zones of the sticklebacks' gastrointestinal tract. The surface of the procercoid consists of several layers that could here be distinguished in the histological sections. The two outer layers are likely most important here, since they are exposed to the host tissue and thus probably involved in host parasite interactions. In the stomach of the stickleback, the outer layer of the procercoid (Fig. 3 A) consists of a thin membrane, which encases the underlying outer level of the tegument and also seems to attach the cercomer to the main body of the procercoid, since no other connection was visible at any plane of the section series. The entire surface of the tegument is covered with microtriches, forming a brush border that is typical for all cestodes (Charles 1971; Mehlhorn 2001; Morris & Finnegan 1969; Smyth 1994; Smyth & McManus 1989).

When still in the stomach, parasites clearly possess the cercomer, thus appearing as a typical procercoid. By contrast, tapeworm stages in the gut, gut wall and body cavity were already plerocercoid-like in appearance, without a cercomer. Already in the anterior part of the gut they neither possessed a cercomer nor were they covered with the outer membrane (Fig. 3 B). Later in the gut wall and in the body cavity the worm's tegument with its microtriches adjoined the host tissues (Fig. 3 C and D).

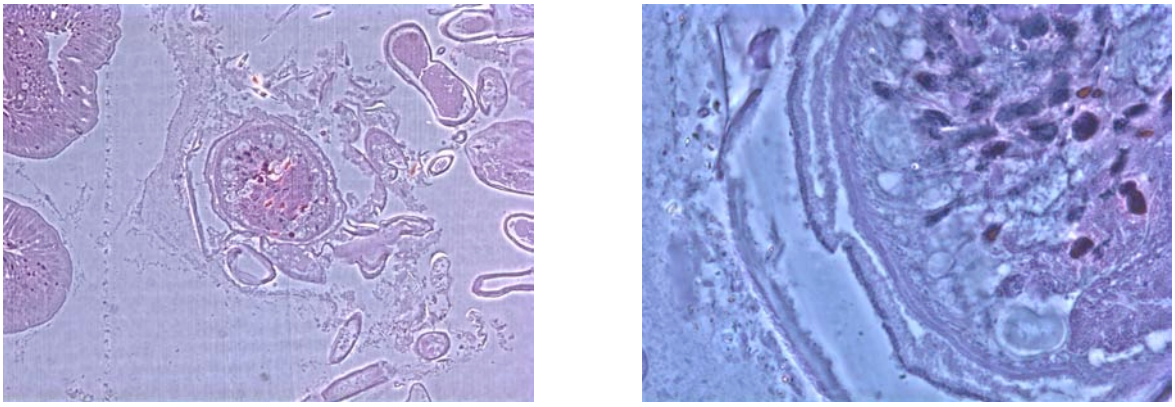


Figure III-4: Micrographs of a histological cross-section of a degenerated S. solidus larva in the intestine of a stickleback. Left: overview (phase contrast, 20x), right: detail (phase contrast, 100x). The tapeworm surface layers are disrupted, with the parasite parenchyma (par) exposed to the host's intestinal enzymes.

Discussion

We here studied chronological and histological aspects of the infestation process of three-spined sticklebacks with procercoid larvae of the tapeworm S. solidus. The majority of the parasites penetrated through the anterior part of the midgut between 14 and 24 hours after exposure to the stickleback. Parasites in the gut lumen had already lost their cercomer together with a thin outer membrane covering the body and cercomer. In contrast to previous assumptions that parasites would loose their cercomer while penetrating the gut wall (Dubinina 1966), these results indicate that the cercomer and outer membrane are lost at an earlier stage, thereby exposing the underlying tegument with its microtriches.

As expected, the percentage of parasites still in the fish stomach decreased significantly over time, while it increased in the body cavity. Next to this general pattern, two aspects are particularly interesting. Firstly, after ingestion, parasites were here observed to stay in the stomach of the fish for at least 12 hours before they appeared elsewhere, which contrasts with other studies, where the only found plerocercoid of S. solidus was recorded in the body cavity already 2 hours post infection (Clarke 1954). This discrepancy might result from differences between the studies in fish and parasite genotype and age, fish hunger level or rearing temperature, as partly discussed in other studies (Dubinina 1966). Secondly, most of the parasites were found either in the stomach or in the body cavity of the stickleback, but not in the gut. This indicates that the migration within the gut and

through the gut wall happens relatively rapidly. Parasites were only found while migrating through the gut tissue in the anterior part of the midgut. In this study, we cannot say if that part is especially easy to penetrate or if the parasite has to escape from the dangerous environment of the gut with its permanent contractions, the risks of enzymatic digestion and an activated immune system.

Procercoids, which we found in the stomach of sticklebacks already two hours post exposure, showed a characteristic organisation of several surface layers that have previously been described in other studies (Dubinina 1966; Morris & Finnegan 1969; Smyth 1994; Smyth & McManus 1989): a thin membrane forms an outer layer that faces the stomach environment and encases an underlying tegument with the microtriches. The outer membrane seems to also attach the cercomer to the body of the parasite. In a previous study, we could distinguish these two separate layers through labelling with lectins that identify the surface sugar composition (Hammerschmidt and Kurtz 2005b; chapter IV). We found the outer layer to mainly consist of PNA-binding sugars (GalNAc, D-galactose), which are supposed to protect underlying parasite tissue from enzymatic digestion (Ingold et al. 2000; Obregón-Henao et al. 2003; Sandemann & Williams 1984). This could also be important in our system, where only in the aggressive stomach milieu tapeworms possessed this outer membrane. Upon probable digestion or shedding of this membrane in the stomach, the cercomer is detached from the body, and the underlying tegument with the microtriches is exposed when the parasite reaches the gut. In the lectin study, this cuticle was found to contain mostly WGA-binding sugars (GlcNAc, sialic acids), which might then help evading the immune system of the stickleback (Hammerschmidt and Kurtz 2005b; Schmidt & Peters 1987; chapter IV).

The present study supports the multifaceted role of different types of microtriches in cestodes, where they are used for the uptake of nutrients, for parasite movement and for keeping the distance and anchoring to the host's tissues (Mehlhorn 2001). In *S. solidus* the microtriches are likely important during gut wall penetration, comparable with the migration of *Taenia* and *Echinococcus* oncospheres (Barker 1970; Heath 1971). This view is supported by the position and orientation of the microtriches, which are larger and denser at the anterior end of the parasite, where the glands that are assumed to be important for host tissue penetration are situated (Dubinina 1966; Smyth & McManus 1989). After reaching the lumen of the

anterior part of the midgut, the tapeworm probably quickly moves towards the gut epithelium, attaches with the microtriches, lyses the epithelial gut layer and migrates through contractions of its body into the lamina propria. Here it possibly migrates through the relatively large intercellular spaces into the body cavity of the stickleback.

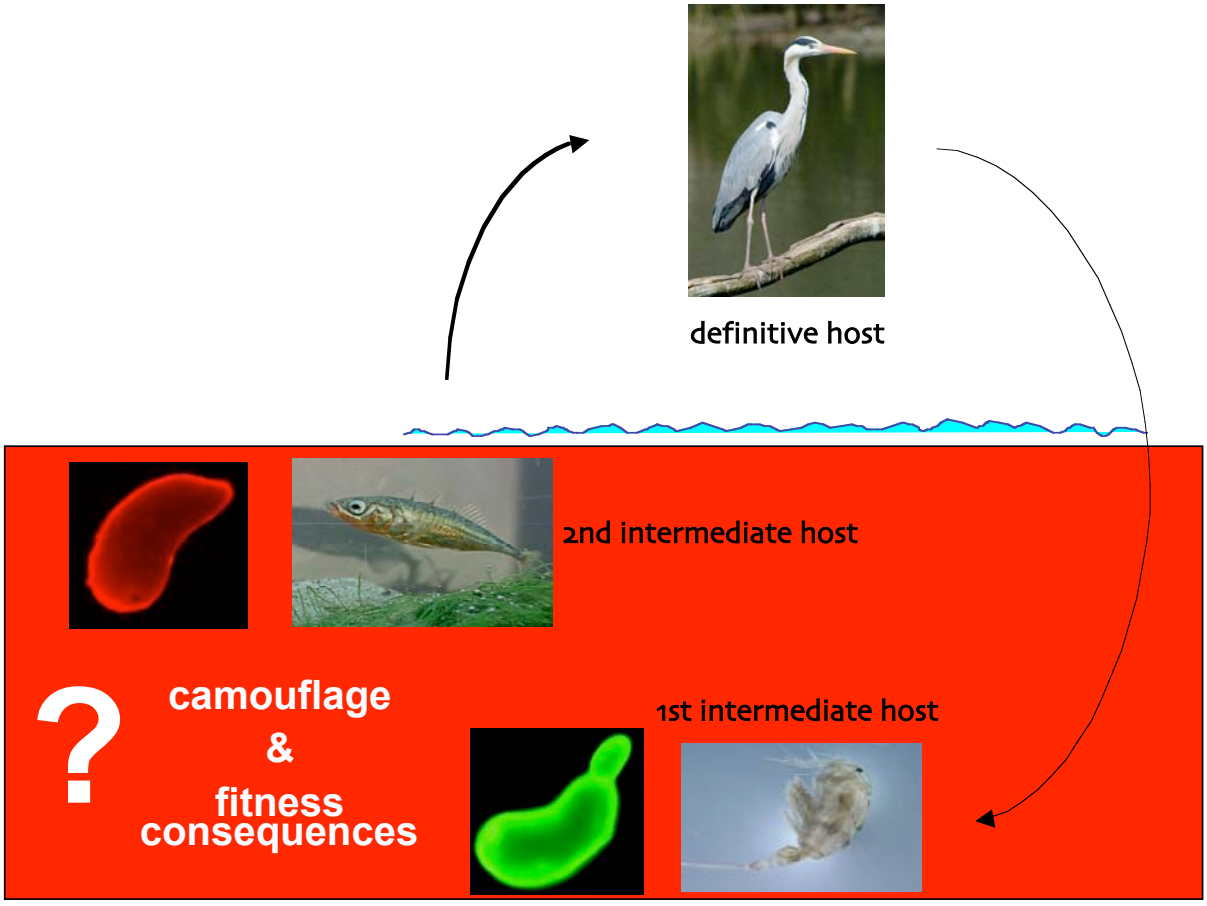
What are the crucial steps of infection and where is scope for the parasite to increase infection success, but then the host to increase resistance? Approximately only one quarter of the parasites are able to overcome the several barriers before reaching the body cavity (Hammerschmidt & Kurtz 2005a; chapter VI). Based on the current study, we can imagine several parasite characteristics that contribute to successful invasion.

Firstly, procercoids have to be fast. After reaching the stomach with its acidic environment, procercoids are only protected by a thin membrane. If damaged, the parasite will likely be vulnerable to digestion. Secondly, the parasite shell should be sufficiently stable to protect against mechanical damage by sharp pieces of food items such as copepod spines. Both digestion and mechanical damage might explain why starved sticklebacks have been found to be easier to infect: an empty digestive tract could accelerate gut passage and reduce mechanical stress. Thirdly, the worms could differ in their ability to attach to the gut epithelium and migrate through the gut tissue (Smyth & McManus 1989). Finally, parasites should be able to survive attack by the host's immune system. Populations of leucocytes (macrophages, lymphocytes, mast cells, granulocytes and mast cells) are present in the gut epithelium and lamina propria of teleost fish (Georgopoulou & Vernier 1986; McMillan & Secombes 1997; Press & Evensen 1999). Strong oxidative burst reactions may especially occur in the gut, where they may be less harmful to host tissue (Read & Skorping 1995). Moreover, strong Th2 responses against certain parasites might only be effective in the gut (Mulcahy et al. 2005). Escape from efficient immune reactions in the gut is even discussed as a possible reason for the evolution of tissue migration in parasitic helminths (Mulcahy et al. 2005; Read & Skorping 1995).

In summary, we here determined key parameters of a crucial phase of infection of sticklebacks with *S. solidus*, the time between ingestion and establishment in the body cavity. We suggest that during this phase, the outcome of infection depends on several traits of both host and parasite, rather than on simply the host's gut wall as a mechanical barrier. Further experiments are needed to analyse more deeply whether

hosts and parasites show variation in these traits that might then be a basis for coevolutionary arms races and moreover constitute a foundation for the development of anti-helminths drugs.

CHAPTER IV


definitive host
2nd intermediate host
?
camouflage
&
fitness
consequences
1st intermediate host

Surface carbohydrate composition of a tapeworm in its consecutive intermediate hosts: individual variation and fitness consequences

Abstract

Carbohydrates on parasite surfaces have been shown to play an important role in host parasite coevolution, mediating host non-self recognition and parasite camouflage. Parasites that switch hosts can change their surface molecules to remain undetected by the diverse immune systems of their different hosts. However, the question of individual variation in surface sugar composition and its relation to infectivity, virulence, immune evasion and growth of a parasite in its different hosts is as yet largely unexplored. We studied such fitness consequences of variation in surface sugars in a sympatric host parasite system consisting of the cestode *Schistocephalus solidus* and its intermediate hosts, a copepod and the three-spined stickleback. Using lectins to analyse the sugar composition, we show that the tapeworm changes its surface according to the invertebrate or vertebrate host. Importantly, sugar composition seems to be genetically variable, as shown by differences among tapeworm sibships. These differences are related to variation in parasite fitness in its second intermediate host, i.e. infectivity and growth. Surface sugar composition may thus be a proximate correlate of the evolutionarily relevant variability in infectivity and virulence of parasites in different hosts.

Introduction

The recognition of molecular patterns that are associated with non-self is a key element for the immunological defence of hosts against pathogens and parasites (Coombe et al. 1984; Janeway et al. 1999; Janeway & Medzhitov 2002). As a basic principle, it involves immunological receptors that identify targets on the surface of the intruders. Host parasite interactions thus essentially take place at the interface between the surface of the parasite and its hosts' immune system. It seems that the surface of all parasites contains carbohydrate (i.e. sugar) residues (Jacobson & Doyle 1996), which are therefore important targets for hosts to distinguish self from non-self. It was shown that carbohydrates, rather than proteins, comprise antigens that dominate the immune response to protozoan and helminth parasites (for review see Gasque 2004; Nyame et al. 2004). Many of these sugars are specifically recognized by receptors of the host immune system, e.g. lectins (Nyame et al. 2004). Lectins are proteins that specifically bind to carbohydrate residues, such as those occurring in glycoproteins of membranes (White 1987). They are also useful tools for parasitologists to monitor parasite surface composition. Such 'lectin labelling' showed that many parasites adapt their surface 'coat' to the host (Ham et al. 1988; Horák 1995; Joachim et al. 1999; Schabussová et al. 2003). This strategy of a parasite to evade its host's immune system became known as 'molecular mimicry' (Damian 1964). Parasites may try to escape or to reduce immune responses of hosts, either in a passive way by masking themselves with host epitopes or actively by mimicking host immuno-regulatory molecules (reviewed in Damian 1997).

Many parasites have complex life cycles, which means that they have to pass through several hosts to reach maturity. Presumably, the parasite surfaces needed to cope with the relatively simple immune system of invertebrates, which often serve as intermediate hosts, will be different to those for coping with the elaborate innate and adaptive immune functions of vertebrates, generally functioning as final hosts (Lucius & Loos-Frank 1997; Smyth 1994). Changeable surface coating may then enable those parasites to evade the immune systems of their different hosts (Damian 1997).

Parasitologists have been very successful in analysing the physiological and molecular mechanisms that parasites use to exploit their hosts (Smyth 1994). These studies are essentially based on the assumption that such adaptation is optimal for the parasite. Evolutionary biologists have taken a different approach in assuming that

there is considerable variation among individual parasites in the traits that lead to adaptation to their hosts (Carius et al. 2001; Little & Ebert 2001). Since there is likewise variation in host counter-measures (i.e. immune defence), this may lead to cycles of adaptation and counter-adaptation, culminating in an arms race that became well known among evolutionary biologist as the 'Red Queen' phenomenon (van Valen 1973; Ebert & Hamilton 1996).

While parasitologists primarily analysed differences in surface structures between developmental stages of a parasite species, evolutionary biologists focussed on traits of parasites that potentially lead to differences in the fitness of parasites in the different hosts (Davies et al. 2001; Gower & Webster 2004; Ham et al. 1988; Horák 1995; Joachim et al. 1999; Schabussová et al. 2003). However, few studies link individual variance in the surface characteristics of a parasite to its ability to infect and exploit its hosts. We bridged that gap by using a multidisciplinary approach and analysed both surface characteristics and fitness of a tapeworm in its two intermediate hosts. To get an understanding of the genetic basis of the traits studied, we further considered variation among genetically related lines by using parasite sibships (i.e. broods).

The host parasite system consists of the cestode *Schistocephalus solidus*, which is confronted with the immune systems of its two intermediate hosts, the copepod *Macrocyclops albidus* and the three-spined stickleback *Gasterosteus aculeatus*. When eaten by a copepod, the parasite, coracidium, penetrates the gut wall and establishes in the body cavity as the procercoid larval stage. After being consumed by a stickleback, it grows as a plerocercoid until it is infective for the definitive host, one of several species of fish-eating birds. Experimentally, we replaced the bird host with an in vitro system for the cultivation of tapeworms (Kurtz 2003; Smyth 1946). Tapeworms seriously reduce the fitness of both intermediate hosts (Arme & Owen 1967; Franz & Kurtz 2002).

The body surface of cestodes is coated with a layer of mucopolysaccharides and glycoproteins, the glycocalyx (Smyth & McManus 1989) but detailed knowledge of sugar composition of the glycocalyx of *S. solidus* is not available. To gain insight into a broad range of potentially relevant surface sugars, we used a mixture of three commercially available, plant-derived lectins that bind to distinct sugars for labelling.

The sugars identified by these lectins have the potential to be important in host parasite interactions (Basseri et al. 2002; Obregón-Henao et al. 2003).

We here analysed whether *S. solidus* changes its surface sugar composition when switching from the copepod to the stickleback host. To explore the fitness consequences of surface composition, we related the variation among genotypes to their performance (i.e. infectivity and growth) in the two hosts. We expected tapeworm performance to be mediated by the hosts' immune reactions and so analysed the activation of the innate immune system and the body condition of the stickleback hosts.

Materials and methods

Parasites

Infected sticklebacks were caught from the brackish 'Neustädter Binnenwasser' (longitude: between 10°40' and 10°50' East, latitude: between 54°59' and 54°60' North), northern Germany, in autumn 2001 and were kept in large tanks with food ad libitum until *S. solidus* tapeworms were dissected from them in February 2003. Sixteen tapeworms (i.e. plerocercoids) were matched pair-wise with regard to size (body weight) to ensure that the worms reproduced by outcrossing (Lüscher & Milinski 2003). The worms were bred in pairs for 6 days in an in vitro system replacing the bird's gut (Smyth 1946; Wedekind 1997). The eggs from each worm pair were divided into 12 portions and stored at 4°C in the dark until use. The offspring from one pair will here be referred to as 'parasite sibship', so that in total eight parasite sibships were used in the experiments. We used sibship as a surrogate for different genotypes and are thus able to determine the upper boundary of heritability. Additional to genetic variation this estimate may also contain common environment effects (Falconer 1981). For three weeks before infection, the eggs were kept at 20°C in the dark. To stimulate the eggs to hatch, they were exposed to light on the day before the infections took place (Dubinina 1966).

Infection of the first intermediate host: copepods

Copepods of the species *Macrocyclops albidus* were kept in culture in the laboratory as described in van der Veen and Kurtz (2002). The culture originated

from 80 individuals from a small river 'Kremper Au' (Neustadt, Germany), which is connected to the 'Binnenwasser', the source of the parasite population.

Thirty days before the start of the experiments, 10 tanks were set up with 50 adult females each. Two days before exposure to tapeworm larva (i.e. coracidium), 1152 adult male copepods were filtered from the culture tanks and randomly assigned to one of the eight parasite sibships. The copepods were singly transferred into a well of a 24-well plate with 2 ml of water and singly exposed to one coracidium each. Thereafter, each copepod was fed ad libitum with five freshly hatched nauplii of *Artemia salina* three times per week. Copepods were kept at 20°C and 16:8 light:dark cycle.

At 10 days post infection, each copepod was screened under the microscope and infection status determined (van der Veen & Kurtz 2002). Parasite screening itself was done without knowledge of parasite sibship. Two days after checking for parasites, infected copepods were dissected for recovery and measurement of the procercoids. The parasite larvae were killed by adding a drop of 20% formalin in phosphate-buffered saline (PBS) to the water. For measurement of procercoid size (i.e. area), an image was taken with a video camera and analysed with the image analysis program Image J 1.31v (Wayne Rasband, National Institutes of Health, USA). From each procercoid, two pictures were taken to check for repeatability of the measurements, which was 99.6 % (calculated using variance components), and the mean of those two measurements was used.

Infection of the second intermediate host: sticklebacks

Before sticklebacks (second intermediate host) can be exposed to the tapeworm, copepods (first intermediate host) have to be infected. Copepods (n=960) were randomly assigned to one of the 8 parasite sibships, singly exposed to one coracidium each and screened for procercoids 12 days post infection.

Stickleback hosts (*G. aculeatus*) were bred in the laboratory from adults, derived from the same 'Binnenwasser' population as the parasites. Offspring from four stickleback pairs, all hatched in July 2002, were raised in family tanks with 15 to 25 fish each. During the experiment, 176 fish were housed in one of two individual compartments of a tank (21 x 35 x 25 cm), without any contact (visual or olfactory). The tanks were randomly distributed across the shelves in the aquaria room (18°C

and 16:8 light:dark cycle). Fish were fed ad libitum three times per week with frozen chironomids.

Two days before exposure to infected copepods, the sticklebacks were transferred into small tanks with 2 L water and starved to enhance consumption of copepods. On the day of exposure, each of the 176 fish was given one copepod that was infected with one parasite larva. Individuals of the different fish families were randomly assigned to the 8 different parasite sibships, and the combination of fish family and parasite sibship was balanced. Two days post infection, the fish were returned to their previous tanks.

One week post infection half of the fish were dissected and the body cavity screened for tapeworms by rinsing the fish tissues in PBS. At this stage, the worms are still translucent and suitable for lectin labelling. The remaining fish were killed 5 weeks post infection. At that time the tapeworms are large enough to determine their weight to the nearest 0.1 mg. On both dissection dates, a hepatosomatic index (I_H) was determined as the weight of the liver divided by body weight, both measured to the nearest 0.1 mg, as 100x liver weight divided by fish weight (Chellappa et al. 1995). I_H is a useful measure of metabolic body condition (i.e. medium term energy reserves) in fish, and was reduced by tapeworm infection in previous studies on sticklebacks (Kurtz et al. 2004).

Immune defence of sticklebacks

To obtain estimates of the activity of the immune system of the sticklebacks, we isolated leucocytes from the head kidneys of freshly dissected fish. We quantified an innate immune reaction that releases reactive oxygen species (ROS) to kill pathogens. This respiratory burst assay measures activity associated with phagocytosis of zymosan particles in vitro in a lucigenin-enhanced chemiluminescence (CL) assay (Scott & Klesius 1981). For this assay, the cell density was adjusted to 1.25 x 10^6 live cells/ml, corresponding to 2 x 10^5 cells/assay (Kurtz et al. 2004). Unfortunately we did not obtain enough cells for the immune assays from all fish so that data of 153 individuals went into the final analyses.

Lectin labelling of parasites

Schistocephalus solidus procercoids and plerocercoids were obtained from experimentally infected copepod and stickleback hosts, respectively, upon dissection. The worms were stored in 250 µl of 4 % formalin in 0.01 M phosphate-buffered saline (PBS), pH 7.4, in a 96 well-plate until lectin labelling. This method of preservation did not change the staining patterns compared to fresh material, except for an increase in autofluorescence (Colditz et al. 2002), which is not relevant in our study since we are interested in relative rather than absolute binding intensities of the samples.

To determine the sugar composition on the surface of individual procercoids and plerocercoids, we used the following lectins, which were labelled with different fluorescent dyes: fluoroscein isothiocyanate (FITC)-labelled PNA (*Arachis hypogaea*) binds to β-galactose-1,3 N-acetylgalactosamine (GalNAc) and D-galactose, tetramethylrhodamine isothiocyanate (TRITC)-labelled WGA (*Triticum vulgaris*) identifies N-acetylglucosamine (GlcNAc) and sialic acid residues (PNA and WGA from Sigma-Aldrich) and Alexa Fluor-labelled ConA (*Canavalia ensiformis*) recognizes α-mannose and β-D-glucose (Molecular Probes) (Jacobson and Doyle 1996). Each tapeworm was labelled with all three dyes simultaneously.

All three lectins were dissolved in 0.05 M Tris-buffered saline (TBS), pH 7.4, to a final concentration of 10 µg/ml. For labelling, parasite larvae were singly transferred with 50 µl of 4% formalin in PBS into a well of a 96 well UV-plate (Corning Incorporated). 200 µl of the lectin mixture was added to each parasite and incubated for 1 h in the dark at room temperature. Parasites were then washed with TBS three times for 10 min. To show that our lectins bind specifically to their ligands we performed controls in which we added a surplus of free sugar with the same binding specificity. Lectins were incubated with their respective inhibitory ligands for 30 min at room temperature in the dark (PNA: 0.2 M β-Lactose monohydrate, WGA: 0.02 M N,N'-Diacetylchitobiose, ConA: 0.2 M α-D-methylmannopyranoside and 0.2 M β-D-methylglucopyranoside) and subsequently used for labelling of procercoids. This procedure successfully blocked the labelling of the procercoids.

Fluorescence microscopy

The parasites were examined using an epifluorescence inverted microscope (Leica DM IRB), using filter sets with emission wavelengths of 515 to 560 nm for the detection of TRITC (absorption/ emission maxima of 555/580 nm), 450 to 490 nm for the detection of FITC (absorption/ emission maxima of 494/518 nm) and 340 to 380 nm for the detection of Alexa Fluor (absorption/ emission maxima of 346/442 nm). With a video camera mounted on the microscope, images were obtained with all three filter settings, digitised and saved in the 8 Bit TIFF-RGB (Red-Green-Blue) mode. To minimise variation in staining due to unequal handling of the parasites, pictures were always taken in the same order of filter settings (TRITC, FITC, Alexa Fluor).

To quantify the intensity of labelling, images were analysed with the image analysis program IP Lab 3.6.2 (For Mac OS 9.2.2, Scanalytics, Inc.). We used the thresholding function to identify the outline of the worm, and then obtained mean intensities of this area (scaled from 0 to 255) of the appropriate colour channel (red for TRITC, green for FITC, blue for Alexa Fluor).

Additional confocal microscopy was used for a more detailed examination of the localisation of the lectins in procercoids, especially in order to identify relevant sugars, such as those at surfaces that are likely exposed to the immune system of the different hosts. We scanned whole lectin-stained procercoids on a Leica confocal laser-scanning microscope TCS SP1.

Data analysis

To check whether developmental stages and sibships of the tapeworms differed in the intensity of lectin labelling, we calculated an analysis of variance (ANOVA) with labelling intensity of each lectin (PNA, WGA, ConA) as the response variable and parasite stage and sibship as effects. To directly compare the relative intensity with which the different lectins bound to the procercoid and the plerocercoid stage, we also calculated the proportional binding of each lectin in relation to all other lectins, e.g. 100 x intensity of WGA/ (PNA + WGA + ConA) gives the ‘relative WGA labelling’.

To check for correlations between the intensity of lectin labelling and fitness parameters of parasite and host, we used mean values per tapeworm sibship and

calculated Spearman Rank correlations (due to the relatively small sample size of eight sibships and the potential non-normality of the data).

For analyses of fish immune activation, only data from infected animals were considered. Immune and fitness data of three fish with extremely swollen and yellow livers were excluded. These fish also produced extreme values in the respiratory burst reaction and hepatosomatic index, which together suggests that they suffered from another infection besides *S. solidus*. Outlier values (in a boxplot: values greater than 1.5 times the spread outside the closest hinge), for the labelling intensities of three procercoids were also excluded since these extreme values indicated irregularities during the labelling procedure (Quinn & Keough 2003).

In general, test statistics refer to two-tailed tests, and we considered effects significant at a level of $P<0.05$. Non-significant interactions were removed from the models. Where necessary, we confirmed that the data allowed the use of parametric statistics (e.g. the ANOVAs) by checking that the residuals obtained from the analyses were normally distributed. Analyses were performed with the JMP Version 5.0.1.2 (SAS ™) software for Macintosh ™.

Results

Variation of parasite surface sugar composition between the developmental stages and among parasite sibships

Labelling intensity of all three lectins varied and could be significantly explained by parasite stage and sibship for PNA (effect of stage: $F_{1, 98} = 2253.05$, $P < 0.0001$ and sibship: $F_{7, 98} = 4.08$, $P = 0.0006$), by parasite stage for WGA (effect of stage: $F_{1, 91} = 19.36$, $P < 0.0001$ and sibship: $F_{7, 91} = 2.06$, $P = 0.0558$ and the interaction between stage and sibship: $F_{7, 91} = 3.09$, $P = 0.0056$), and for ConA (effect of stage: $F_{1, 98} = 824.26$, $P < 0.0001$ and sibship: $F_{7, 98} = 1.09$, $P = 0.37$). The labelling was generally more intense for procercoids than plerocercoids (Fig. 1).

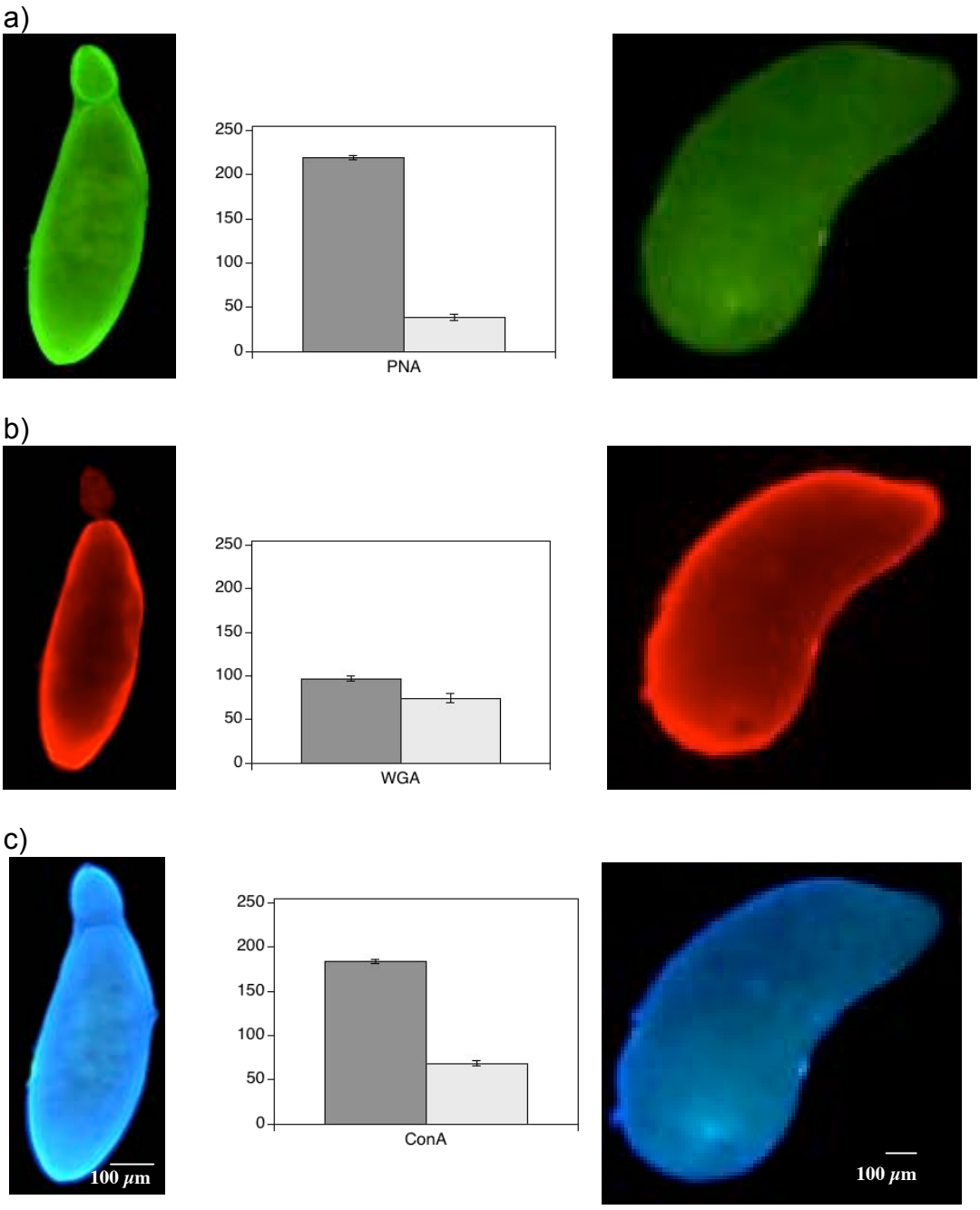


Figure IV-1: Epifluorescent micrographs of a procercoid of *Schistocephalus solidus* (left), dissected from an infected copepod and of a plerocercoid (right), dissected from an infected stickleback. The tapeworms were labelled with three fluorescent lectins: (a) PNA (fluoroscein isothiocyanate labelled labelled), (b) WGA (tetramethylrhodamine isothiocyanate labelled), (c) ConA (Alexa Fluor 350 labelled). Mean intensity of labelling with each lectin is shown for both developmental stages in the bar chart in the middle (left bar: procercoid, n = 77; right bar: plerocercoid, n = 31).

It is thus useful to compare relative, in addition to absolute, intensities of labelling, i.e. to evaluate how strongly the surface of each stage is dominated by one sugar as compared to the others (Fig. 2). The surface of procercoids was dominated by sugars

that are recognized by PNA ($F_{1, 105}$ = 931.17, P < 0.0001), whereas plerocercoids were characterised by WGA-binding sugars ($F_{1, 105}$ = 541.62, P < 0.0001) and the relative intensity of ConA binding did not differ between the stages ($F_{1, 105}$ = 1.12, P = 0.29).

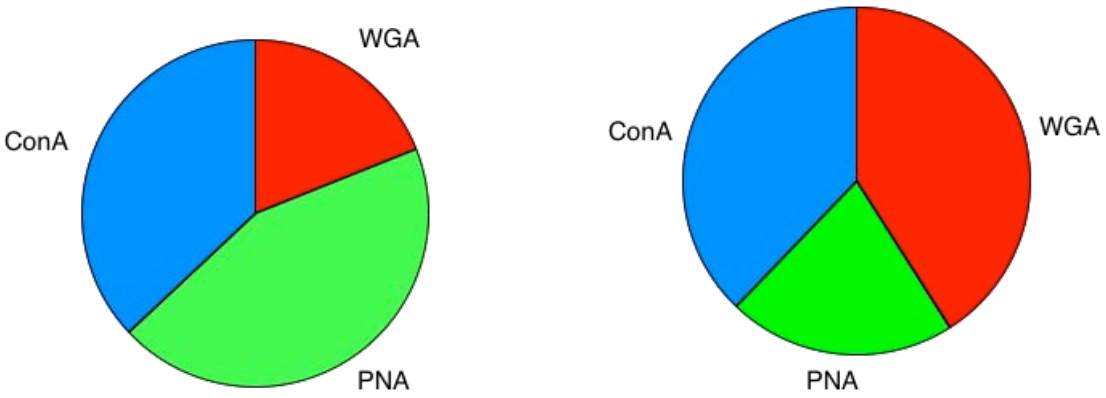


Figure IV-2: Relative lectin binding of PNA (green), WGA (red) and ConA (blue) for the (a) procercoid (n = 77) and the (b) plerocercoid stage (n = 31).

Localisation of lectin binding

In the procercoid, PNA stained the surface layer (Fig. 3a), which encases the WGA-labelled inner layer (Fig. 3b). By contrast ConA labelled the surface layer, but also led to homogenous staining of most interior parts of the body (Fig. 3c). Since host immune effectors will have access to the exposed surface of the parasite, PNA might arguably be considered the most relevant label for the procercoid stage within the copepod.

As there is evidence that the procercoid looses its cercomer (head-like appendage at one side of the body with still unknown function; see also in Fig. 3) upon metamorphosis to the plerocercoid in the fish (Dubinina 1966), it necessarily also looses the outer surface layer (Fig. 3), so that the WGA-binding inner surface will be exposed here. WGA-labelling carbohydrates of the plerocercoid might be the most relevant with regard to exposure to the fish immune system.

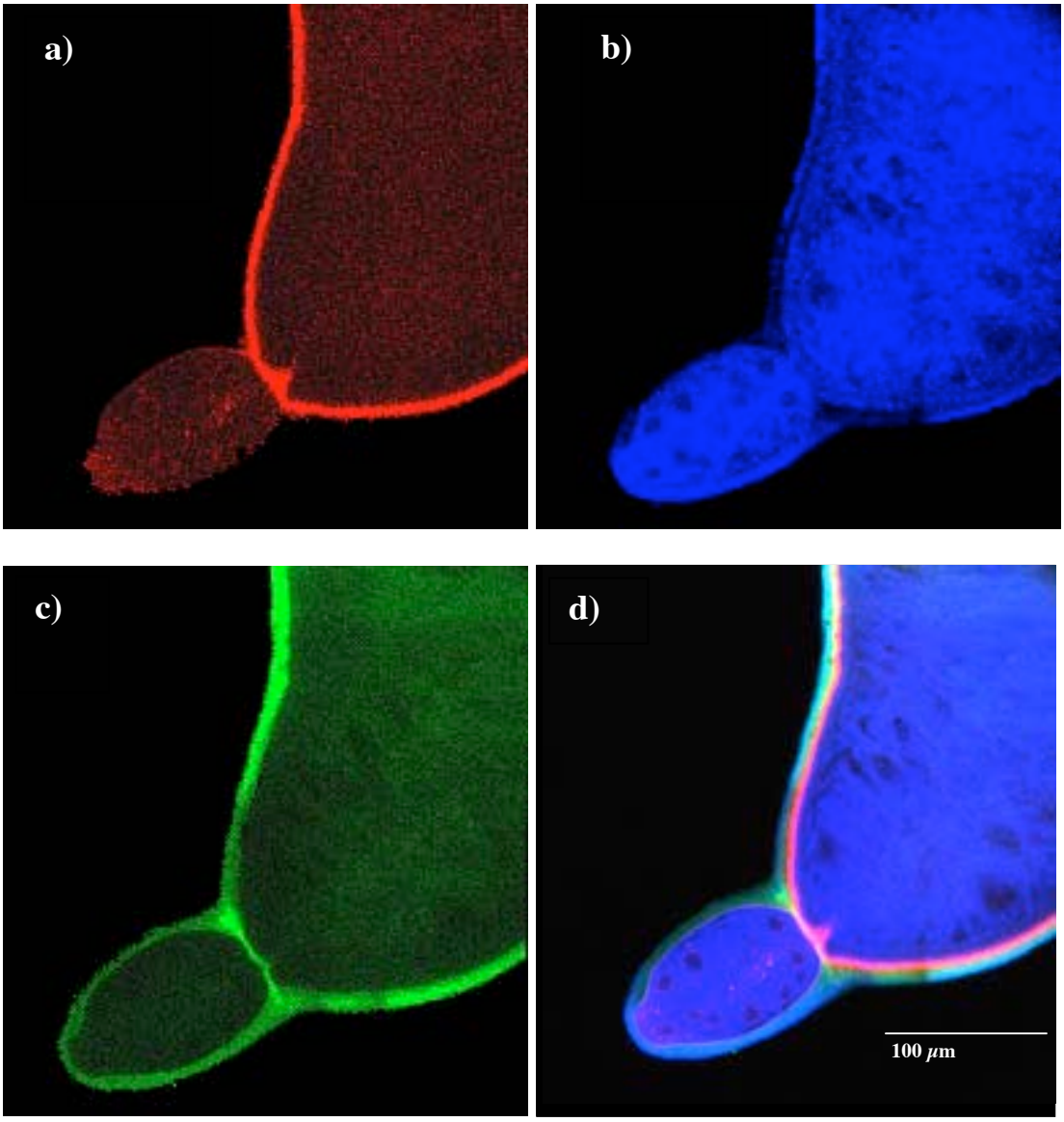


Figure IV-3: Micrographs (confocal microscopy) of a procercoid of *S. solidus*, dissected from an infected copepod, and labelled with different fluorescent lectins: (a) PNA (fluoroscein isothiocyanate labelled), (b) WGA (tetramethylrhodamine isothiocyanate labelled), (c) ConA (Alexa Fluor 350 labelled) and (d) composite image. Generally, the micrographs were taken as single plane images in the same plane and then merged to the composite image for better visualization.

Lectin-labelling in relation to fitness parameters of the parasite and its hosts

We did not find a significant relationship between the mean intensity of PNA binding to procercoids and the mean infectivity of a sibship in the copepod (r_s = 0.14, N = 8, P = 0.73), nor did we find any correlation with mean intensity of PNA binding and the mean size of the procercoids (r_s = -0.11, N = 8, P = 0.77).

For the plerocercoid stage, the mean labelling intensity of WGA of a tapeworm sibship correlated negatively with its infectivity in the fish (r_s = -0.82, N = 8, P = 0.01). By contrast, the correlation with the plerocercoid body weight was positive (r_s = 0.75, N = 8, P = 0.05) (Fig. 4a, b).

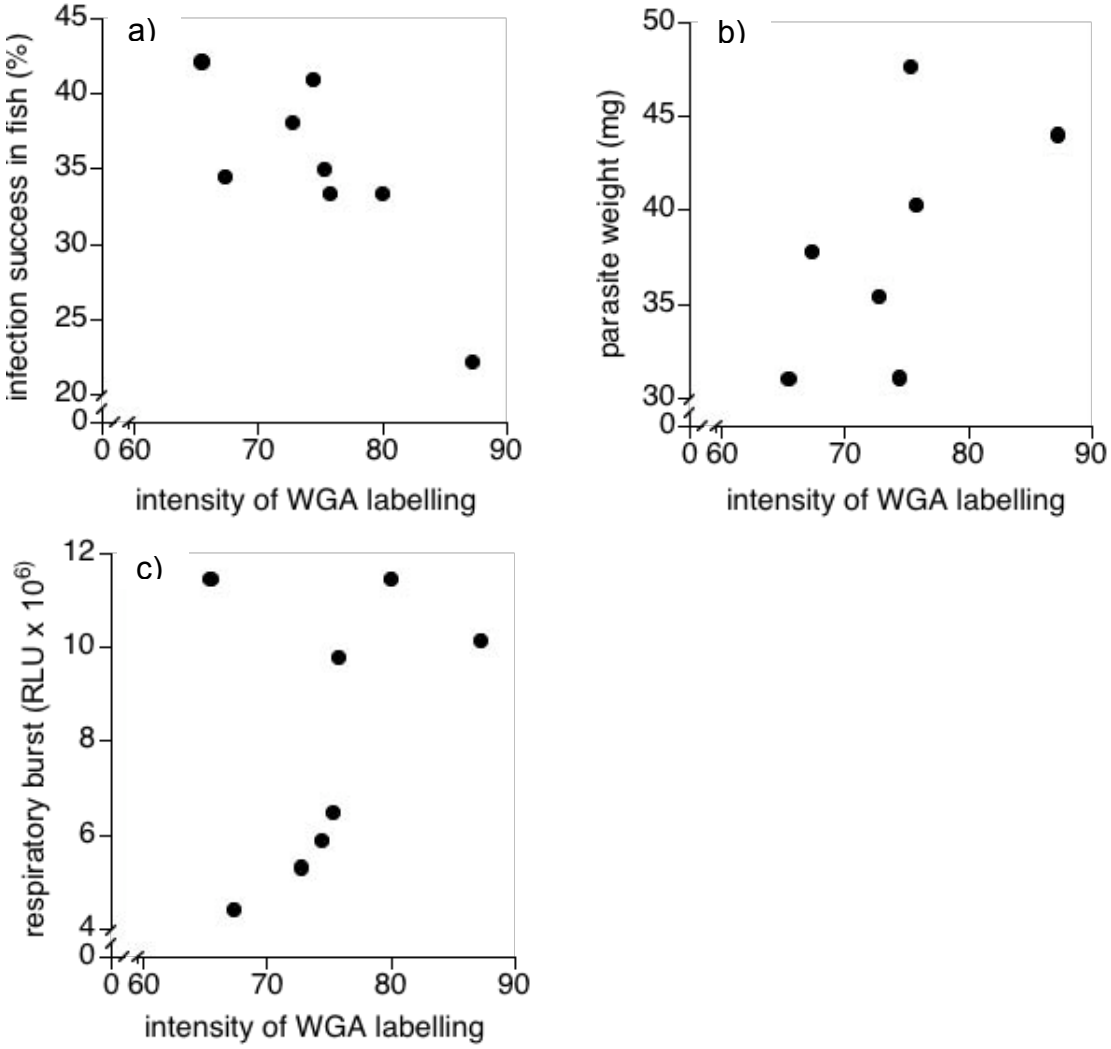


Figure IV-4: Relationship between the mean WGA labelling intensity of plerocercoids of the tapeworm *Schistocephalus solidus* and (a) infectivity (%) in the fish, (b) plerocercoid weight (35 days post infection) and (c) stickleback innate immune response (respiratory burst 7 days post infection). Data points show the mean of a parasite sibship (2 to 6 individuals).

The observed associations between the labelling characteristics and infectivity of a worm sibship could not significantly be explained by differential activation of the stickleback immune system, as estimated from the respiratory burst reaction (correlation of the respiratory burst with procercoid-PNA: r_s = -0.14, N = 8, P = 0.73; with plerocercoid-WGA: r_s = 0.30, N = 8, P = 0.45) (Fig. 4c). Likewise, there was no

detectable link between these tapeworm surface characteristics and the metabolic condition of the infected sticklebacks (correlation of the hepatosomatic index I_H with procercoid-PNA: r_s = 0.09, N = 8, P = 0.82; with plerocercoid-WGA: r_s = -0.33, N = 8, P = 0.41).

Discussion

Using lectin labelling to analyse carbohydrates, we found the surface of the tapeworm *S. solidus* to be highly glycosylated. The relative proportion of surface sugars varied between the two developmental stages, the pro- and plerocercoid. Importantly, sugar composition seems to be genetically variable, as shown by differences between tapeworm sibships. These differences relate to variation in parasite fitness, i.e. infectivity and growth in the second intermediate host, the stickleback. We thus conclude that the differing ability of individual parasites to adapt their surface to their consecutive intermediate hosts leads to evolutionarily relevant variation in fitness. However, with the rather simple measures of host immune activity that were analysed here, we could not identify the immunological basis for such variation.

How did the surface sugar composition vary between the two developmental stages of the parasite? In the procercoid stage, which has to deal with the immune system of an invertebrate (a copepod), relatively more PNA-binding sugars (GalNAc, D-galactose) were found, whereas in the plerocercoid stage, which has to evade the immune system of a vertebrate (the stickleback), relatively more WGA-binding sites (GlcNAc, sialic acid residues) were identified.

In our study, lectin labelling thus shows the surface of the procercoid to be dominated by GalNAc and D-galactose. This is in line with several reports in the literature, where such sugars were mainly found on the surface of parasite larvae in invertebrate hosts. It was reported that these sugars are important in interactions between insect vector tissues and their parasites (Burton et al. 1999; Knowles et al. 1991). In the case of the nematode *Onchocerca lienalis*, the parasite is thought to mimic host sugars not be recognised as foreign by the invertebrate host's immune system (Jacobson & Doyle 1996). However, PNA-binding sugars were also hypothesized to protect underlying tissues of parasites from enzymatic digestion in

the intestines of their vertebrate hosts (Ingold et al. 2000; Sandeman & Williams 1984; Obregón-Henao et al. 2003). Procercoids of *S. solidus* also face the harsh environment of the stomach and gut of the stickleback before penetrating through the gut wall into the body cavity. The high intensity of PNA-binding sugars on procercoids of *S. solidus* could therefore function as a protection in both, the invertebrate and vertebrate host.

Within each stage of the parasite, we analysed the variance of surface sugar composition among tapeworm sibships, which we correlated to their average fitness. For the procercoid stage in the copepod host, we did not find any correlations of lectin binding and fitness. A possible explanation could be that the surface of the oncosphere, which is exposed to the copepod immune system shortly after infection, rather than the procercoid, is mostly relevant for the interaction with the copepod immune system (Dubinina 1966; van der Veen & Kurtz 2002). Due to technical problems in removing the outer layer of the coracidia, we could not label oncospheres.

On the surface of the plerocercoid stage in the fish host, we mainly found WGA-binding sugars, which were previously reported for other parasites (Fuchs et al. 1999; Jacobson & Doyle 1996; Obregón-Henao et al. 2003). WGA recognizes both GlcNAc and sialic acid residues, but a more detailed analysis would be needed to distinguish them. Sialic acids have so far rarely been reported for helminth parasites (but see Maizels et al. 2004). Arguably, sialic acid residues on the surface of *S. solidus* could help evading the immune system of the stickleback, since all vertebrate cells are covered with terminal sialic acids, whereas the absence of sialic acids, e.g. on parasite surfaces, is known to act as a non-self signal (Maizels et al. 2004; Schauer 2004).

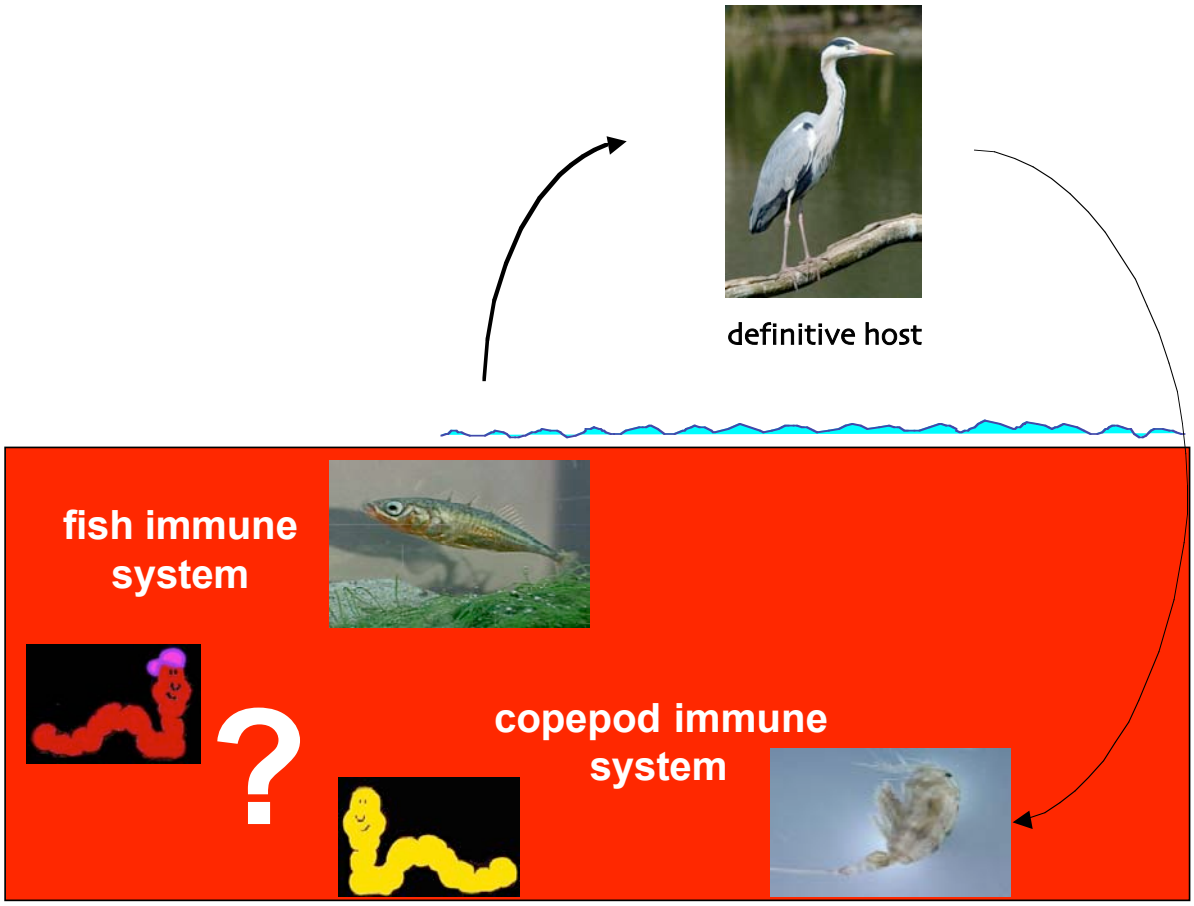
In the stickleback, we found that parasite sibships that bound more WGA were inferior in infecting their host, but superior when it came to increasing their body size. Size is important for worm fitness, since it directly relates to egg production (Wedekind 1997). Plerocercoids seem to face different parts of the immune system at different time-points of infection: in a recent study, tapeworm growth was for example found to be regulated by components of the adaptive immune system (Kurtz et al. 2004). Tapeworms may cope best with either the innate part of the defence system (thus being superior in infecting the stickleback) or with adaptive immunity

(thus growing bigger later during an established infection). WGA-binding sugars, which may protect from adaptive immunity but seem more prone to detection by the innate defence system, could mediate such a trade-off (Hammerschmidt and Kurtz 2005a; chapter V). Parasite surface sugars can even play a more active role, such as immuno-suppression or immuno-avoidance (Ma et al. 2002; Plows et al. 2005; Tomaska & Parish 1981). There are indications for suppression of the stickleback immune system by *S. solidus* during later stages of the infection (Scharsack et al. 2004).

We here made use of plant lectins, which have been used in other studies to specifically detect parasite surface carbohydrates (Jacobson & Doyle 1996; Joachim et al. 1999; Schabussová et al. 2003). Lectins also occur in animals, where they fulfil an important role in the innate immune system (Epstein et al. 1996; Fujita 2002; Turner 1996). Since the interaction of lectins with their sugars is specific, lectins may enable specific recognition of pathogens within the innate immune system. Especially among invertebrates, there are indications that multiple lectins might mediate non-self recognition (Marques & Barracco 2000; Wilson et al. 1999; Zhang et al. 2004). In our host parasite system, it was shown that the copepod is capable of specific immunological memory (Kurtz & Franz 2003). The current study points towards variation in tapeworm surface sugar signatures as potential targets for such specific recognition (Kurtz 2005).

In summary, we found that surface glycoconjugates appear to play an important role in the interaction of the cestode *S. solidus* with its intermediate hosts. Our results match findings in other host parasite systems, where parasites also change their surface coat depending on the host and so evade the hosts' immune systems. Most importantly, we found that stage-specific differences in the surface sugars among individuals in the second intermediate host are associated with important aspects of the fitness of these tapeworms. Surface sugar composition may thus be a proximate correlate explaining variance in infectivity and performance of that parasite in its different hosts. It seems that parasites may use different strategies within their host species to evade diverse immune systems.

CHAPTER V



Evolutionary implications of the adaptation to different immune systems in a parasite with a complex life cycle

Abstract

Many diseases are caused by parasites with complex life cycles that involve several hosts. If parasites cope better with only one of the different types of immune systems of their host species, we might expect a trade-off in parasite performance in the different hosts, that likely influences the evolution of virulence. We tested this hypothesis in a naturally co-evolving host parasite system consisting of the tapeworm *Schistocephalus solidus* and its intermediate hosts, a copepod, *Macrocyclops albidus*, and the three-spined stickleback *Gasterosteus aculeatus*. We did not find a trade-off between infection success in the two hosts. Rather, tapeworms seem to trade-off adaptation towards different parts of their hosts' immune systems. Worm sibships that performed better in the invertebrate host also seem to be able to evade detection by the fish innate defence systems, i.e. induce lower levels of activation of innate immune components. These worm variants were less harmful for the fish host likely due to reduced costs of an activated innate immune system. These findings substantiate the impact of both hosts' immune systems on parasite performance and virulence.

Introduction

In many theoretical models, virulence is assumed to be a major factor that shapes host parasite coevolution. Within the field of evolutionary biology, virulence is defined as the reduction in host fitness following parasitic infection (Hansen & Koella 2003). Since parasites exploit their hosts, such reduction can be seen as an unavoidable side effect of growth or multiplication of parasites. Therefore, virulence is closely connected with parasite fitness. Moreover, reduction in host fitness can also result from the activation of the host immune system. Immunity has been shown to be costly, owing to energetic demands and immunopathological effects (Moret & Schmid-Hempel 2000).

The question as to which factors influence the evolution of virulence, especially which factors restrict virulence is of crucial importance to basic research as well as applied issues and has been addressed in many theoretical studies (Bull 1994; Day 2002; Frank 1996). However, these models concentrate on the evolution of virulence in single-host systems (Frank 1996; Galvani 2003), even though many parasites, of medical and veterinary relevance, have complex life cycles that involve several host species (Parker et al. 2003a). A recent theoretical study (Gandon 2004) stresses that for understanding the evolution of virulence in complex life cycles, more factors need to be taken into account, especially potential constraints among different parasite traits, both between and within hosts. It is likely that optimal exploitation of one host species leads to a reduced ability to exploit another host. The expected negative correlation in the fitness of a parasite in the different host species could genetically be based on antagonistic pleiotropy, since a gene that enhances fitness in one host could decrease fitness in the other host. In one of the few empirical studies in this field, Davies et al. (2001) found a trade-off in the reproductive success in *Schistosoma mansoni* across the mammalian and molluscan hosts. Further evidence comes from serial passage experiments (SPEs), where parasites are subsequently cycled through one host species (for more detail see Ebert 1998). In SPEs virulence normally increases when pathogens are passaged through one host species but may decrease when intermediate hosts are included again (Ebert 1998).

These studies rarely include the physiological factors that may affect the evolution of parasite virulence such as host immunity. Host immunity can be regarded as a double-edged sword. On the one hand, immunity can restrain parasite growth or

multiplication and thereby reduce virulence. On the other hand, immune reactions themselves are costly to produce, not only because they need energy, but also because they may harm the hosts' own tissues (cf. immunopathology) (Moret & Schmid-Hempel 2000; Rolff & Siva-Jothy 2003; Sheldon & Verhulst 1996). Thus, virulence will also depend on the type and degree of host immune responses and their pathological side effects.

In one of the few studies that considers host immunity as a selection pressure that may influence virulence, (Mackinnon & Read 2004) recently showed that parasite sibships of *Plasmodium chabaudi* became more virulent when they evolved in immunized as compared to naïve mice. Multi-host life cycles consist of several invertebrate and vertebrate hosts, all with different immune systems. The divergent activation of these immune systems by the parasite may shape and constrain virulence because multi-host parasites may be forced to trade-off between different immune evasive strategies.

In the current paper, we provide a test of this hypothesis using a natural co-evolved host parasite system. As such, our study contrasts with previous work (Davies et al. 2001; Gower & Webster 2004), in which fitness parameters were measured in parasites adapted to laboratory conditions for many generations. We rather used parasites and hosts that co-occurred in a natural population. Our parasite, the tapeworm *Schistocephalus solidus* is confronted with the immune systems of the copepod *Macrocyclops albidus*, which is a tiny crustacean, the stickleback fish *Gasterosteus aculeatus* and, as a final host, any species of fish-eating bird. Since the intermediate hosts are the major source for the uptake of resources and are used as a vehicle to reach the next host, an infection with *Schistocephalus solidus* leads to serious fitness reduction in both intermediate hosts (Arme & Owen 1967; Kurtz & Franz 2003; van der Veen & Kurtz 2002; Wedekind & Milinski 1996). Selection pressure on defence mechanisms in the host is thus expected to be high and the cestode will therefore be confronted with the problem of coping with competent immune systems in both hosts. We examined whether individuals of *S. solidus* are genetically constrained in their ability to evade the crustacean and the fish equally well. For this test, we used sibships of tapeworms, as a surrogate of different genotypes, and analysed their performance in both hosts using infectivity and growth as fitness parameters. We expected a trade-off in

parasite fitness in the first and the second host, i.e. those sibships that perform well in the copepod might have disadvantages in the fish. Since we suspected performance in the hosts to be mediated through host immune responses, we further analysed parameters that are indicative of the activation status of the immune system of the stickleback host. We also measured the body condition of the fish as an estimate of the damage to the host after infection, to obtain an approximation of the virulence of the parasites.

Materials and methods

Study system

To complete its life cycle, the tapeworm *S. solidus* needs two intermediate hosts, a cyclopoid copepod and the three-spined stickleback *G. aculeatus*, and one definitive host, which is any species of fish eating bird. Infestation of intermediate and definitive hosts occurs through ingestion of the parasite or infected intermediate host, respectively. The worms grow only in the two intermediate hosts, but reproduce during a short time span of several days within the birds' gut (Clarke 1954).

Parasites

S. solidus tapeworms were dissected in February 2003 from sticklebacks, which were caught during autumn 2001 from the brackish 'Binnenwasser' near Neustadt, northern Germany, and thereafter kept in large tanks with food *ad libitum*. The worms (i.e. plerocercoids) were bred in an *in vitro* system that replaces the bird's gut (Smyth 1946; Wedekind 1997). Worm pairs were matched by body weight to guarantee outcrossing (Lüscher & Milinski 2003). Offspring from each pair will here be referred to as 'parasite sibship'. A total of eight sibships were used for the experiments (for details on the experimental design, see Figure 1). Eggs were stored at 4°C in the dark. For hatching, eggs were transferred to 20°C for three weeks and then exposed to light on the day before usage (Dubinina 1966).

Hatching success was determined three month after exposure to light, to allow all viable larvae to hatch. To this end, the proportion of eggs with open or removed operculum was determined in 100 eggs per sibship (Schjørring 2004; Swiderski 1994).

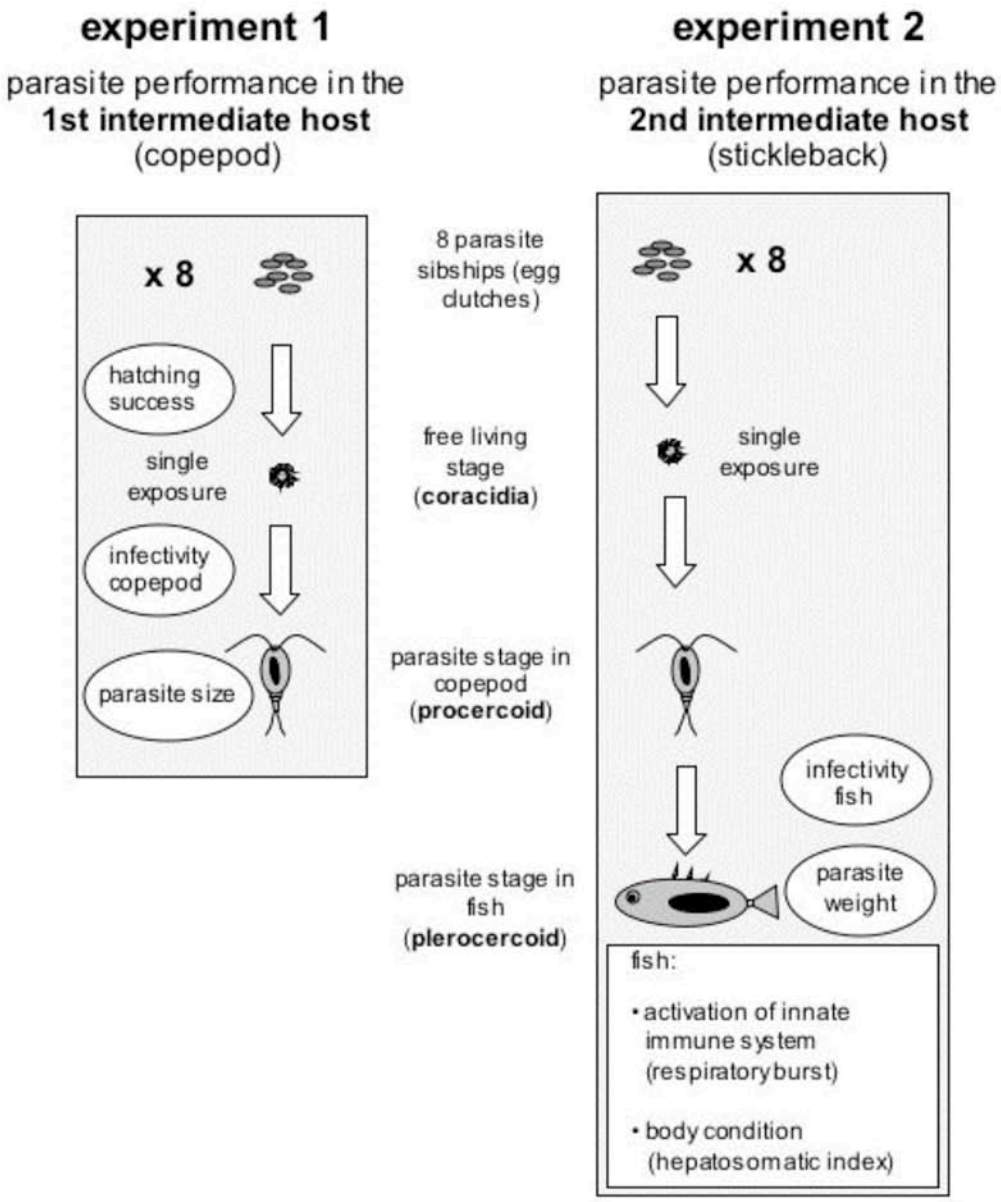


Figure V-1: Experimental design of the experiments. Measures obtained from the tapeworms are shown inside ovals, stickleback traits in a square.

Infection of the first intermediate host: copepods

Macrocyclops albidus copepods were kept in culture in the laboratory as described before (van der Veen & Kurtz 2002). The culture originated from 80 individuals from a small river 'Kremper Au' (Neustadt, Germany), which is connected to the 'Binnenwasser', the source of the parasite population. 30 days before the start of the experiments, 10 tanks were initiated with 50 adult females each.

Two days before exposure to one tapeworm larva (i.e. coracidium) each, 1152 adult male copepods were filtered from the culture tanks and transferred into individual wells of 24-well plates with 2 ml of water. Thereafter, each copepod was fed *ad libitum* with 5 freshly hatched nauplii of *Artemia salina* three times per week.

Copepods were kept at 20°C and 16:8 light:dark cycle. Ten days post infection, infection status of each copepod was determined microscopically, without knowledge of parasite sibship (van der Veen & Kurtz 2002).

Two days after checking for parasites, infected copepods were measured (van der Veen 2003) and dissected to obtain the procercoids, which were killed by adding to the water a drop of 20 % formalin in phosphate-buffered saline (PBS). For measurement of procercoid size (i.e. area), an image was taken with a video camera and analysed with the image analysis program Image J 1.31v (Wayne Rasband, National Institutes of Health, USA). From each procercoid, two pictures were taken to check for repeatability of the measurements, which was 99.6 % (calculated using variance components), and the mean of those two measurements was used.

Infection of the second intermediate host: sticklebacks

For infection of sticklebacks, *S. solidus* infected copepods were obtained as described above, with the exception that, instead of adult males, young copepod stages were used, which are more susceptible (van der Veen & Kurtz 2002). This reduces selection that might occur in the first intermediate host. Such selection could be relevant, since from clutches that perform bad in the copepod host, only the relatively best genotypes might manage to reach the fish host, which could lead to a non-random representation of parasite qualities in the fish host. Infection of the 960 copepods was screened twelve days post infection.

Sticklebacks *G. aculeatus* hosts were bred in the laboratory from adults, derived from the same 'Binnenwasser' population as the parasites. Offspring from four stickleback pairs, all hatched July 2002, were raised in family tanks with 15 to 25 fish each. During the experiment, 176 fish were housed in one of two individual compartments of a tank (21 x 35 x 25 cm), without any contact (visual or olfactory). The tanks were randomly distributed across the shelves in the aquaria room (18°C and 16:8 light: dark cycle). Fish were fed *ad libitum* three times per week with frozen chironomids.

Two days before exposure to infected copepods, stickleback weight (to the nearest 0.1 mg) and size (from the snout to the base of the tail, to the nearest mm) were determined and a condition factor (cf) (Frischknecht 1993) calculated as cf = 100 x W/L^b (fish weight W in g, fish length L in cm and the exponent from the linear regression analysis b = 2.495 of log-transformed values of W and L). Fish were transferred into small tanks with 2 l water and starved to enhance consumption of copepods. On the day of exposure, each fish was given one copepod that was infected with one parasite larva. Individuals of the different fish families were randomly assigned to the 8 different parasite sibships, and the combination of fish family and parasite sibship was balanced. Two days post infection, the fish were returned to their larger tanks.

One week post infection half of the fish were dissected and the body cavity screened for tapeworms by rinsing tissues in PBS. At this stage, the worms are still translucent and very small. The parasites were fixed and measured as described for the procercoids. However, repeatability of this measure turned out to be low, due to the flexible shape of the small plerocercoids. We thus did not consider this measure for further analyses. Remaining fish were killed 5 weeks post infection. At this time the tapeworms are much bigger and their weight could be determined to the nearest 0.1 mg. We also determined again fish length, weight and condition. Moreover, liver weight was determined (to the nearest 0.1 mg) and a hepatosomatic index (I_H) calculated as 100x liver weight/fish weight. I_H is a correlate of medium term energy reserves, thus a good measure of fish metabolic body condition (Chellappa et al. 1995).

Immunity of sticklebacks

We analysed the activity of the innate immune system of the sticklebacks, using leucocytes from the head kidney, which is the major immune organ of bony fish (Zapata et al. 1996). Unfortunately, we did not possess a method to assess the adaptive immune system at the time of the experiment.

Leucocytes were processed as described in (Kurtz et al. 2004) with the following modifications: during washing of the cells, centrifugation was performed at 550 g for 10 min. at 4°C. Differential cell counts were obtained on a Becton Dickinson FACSCalibur ™ flow cytometer using the CellQuest Pro 4.02 software for acquisition

and analysis. All samples were supplemented with propidium iodide (2 mg/l, Sigma Aldrich) to detect dead cells. Forward- and sidescatter values (FSC/SSC characteristics) of at least 10 000 cells were acquired in linear mode; fluorescence intensities at wavelengths of 530 and 585 nm were acquired at log-scale. Cellular debris with low FSC characteristics and dead cells (propidium-iodide-positive) were excluded from further evaluation. Different cellular subsets were identified according to their characteristic FSC/SSC profiles cf. Scharsack et al. (2004), and denoted as lymphocytes (low FSC/low SSC) and granulocytes (high FSC/high SSC). For the adjustment of cell numbers for the subsequent *in vitro* respiratory burst assay, absolute cell counts were determined with the standard cell dilution assay (SCDA, (Pechhold et al. 1994) in a modified form, by addition of 2 x 10^5 green fluorescent particles (4 µm, Polyscience, USA) to each tube, as a standard for counting (Scharsack et al. 2004).

As a functional estimate of innate immune activity, we quantified the respiratory burst reaction. During the respiratory burst, reactive oxygen intermediates are generated to kill pathogens. We analysed the respiratory burst (relative luminescence units (RLU)) associated with phagocytosis of zymosan particles *in vitro* in a lucigenin-enhanced chemiluminescence assay (Scott & Klesius 1981). For this assay, the cell density was adjusted to 1.25 x 10^6 live cells per ml, corresponding to 2 x 10^5 cells per assay (Kurtz et al. 2004). Unfortunately we did not obtain enough cells for the immune assays from all fish so that data of 153 individuals went into the final analyses.

Heritability and covariance between parasite traits

For the estimates of heritability and genetic correlation between traits, an analysis of full-sib families (parasite sibships) was performed. Such designs only give an upper estimate of heritability, because dominance variance and common environment effects cannot be excluded (Falconer & Mackay 1996; Roff 1997). In our system, the latter is assumed to be negligible because once inside their host, siblings do not encounter a common environment any more. To estimate the heritability for the continuous and threshold traits, we performed an ANOVA (analysis of variance) and calculated the standard errors for unequal family size as described in Roff (1997).

When assessing the covariance between traits in full-sib family correlations, the 'family' component of variance contains additive, non-additive and interaction genetic covariance plus maternal covariance, which cannot be separated (Falconer & Mackay 1996; Roff 1997; Via 1984). Thus, all correlations computed here are genetic correlations in a broad sense, which can serve only as a first step towards a more precise estimation.

Some traits are measured in different hosts (environments). Therefore, the analysis cannot be performed on the same individual. We applied an approach, first suggested by Via (1984), where the genetic correlation can be estimated by using the Pearson product-moment correlation between family means (also suggested by Roff 1997). However, we preferred to calculate a more conservative test, the Spearman rank correlation, to account for the relatively small sample size of eight sibships, and the potential non-normality of the data.

Data analysis

We checked for differences in infectivity of the parasites in the two intermediate hosts with log linear models (all effect variables mentioned were included in one model for each host). To analyse which factors influenced parasite size, an ANOVA contained parasite size in the copepod as response and parasite sibship and copepod size as effect variables. For parasite size in the fish an ANOVA was calculated with worm weight as the response and parasite sibship, fish family, fish gender and fish weight before infection as effect variables.

Only data from infected animals were considered in subsequent analyses. Immune and fitness data of two fish had to be excluded: their livers were extremely swollen and yellow and they also produced extreme values in the respiratory burst reaction and hepatosomatic index. This jointly suggested that besides *S. solidus* they suffered from another illness. In general, all interactions were insignificant and thus removed from the models, except for the analysis of the parasite size in the fish where we did not include interactions due to saturation of the model. All test statistics refer to two-tailed tests. We considered effects significant at a level of $P < 0.05$. Analyses were performed with the JMP Version 5.0.1.2 (SAS ^{TM}) software for Macintosh ^{TM}.

Results

Heritability and covariance between parasite traits

Three heritability estimates obtained from full-sib analysis were significantly larger than zero (Table 1). Heritability of infection success and size of the parasites in the copepod host was 0.65 and 0.29, respectively, which indicates a considerable additive genetic component to the phenotypic variance. Genetic (full-sib) correlations between traits within and across hosts are shown in Table 2 and addressed in more detail below.

Table V-1: Broad sense heritability estimates (h^2 ± SE) for parasite traits in the two intermediate hosts. Sample size (n) and number of sibships (N) are given. Estimates that are significantly larger than zero are indicated in bold type.

	trait	full-sib h^2	n	N
	hatching	0.00 ± 0.00	800	8
copepod (procercoid)	**infectivity**	**0.65 ± 0.01**	**1072**	**8**
	size	**0.29 ± 0.01**	**332**	**8**
fish (plerocercoid)	infectivity	-0.09 ± 0.01	170	8
	weight	-0.35 ± 0.04	21	7
	respiratory burst	**0.17 ± 0.04**	**27**	**8**
	hepatosomatic index	-0.28 ± 0.02	29	8

Fitness of tapeworms in their first intermediate host, the copepod

In total, 1072 male copepods survived the experiment and were included in the analysis. Mortality of copepods (0.8%) did not depend on the parasite sibship they had been exposed to (Likelihood Ratio (LR) χ_7^2 = 3.098, P = 0.88). On average, 39.83% of the copepods were infected. The infection rate varied significantly among parasite sibships (LR χ_7^2 = 110.61, P < 0.0001). Body size of the tapeworm larvae in the copepod (i.e. procercoid size) was significantly influenced by parasite sibship and by copepod size (effect of sibship: $F_{7, 323}$ = 8.20, P< 0.0001 and copepod size: $F_{1, 323}$ = 8.52, P = 0.0038). Worms that originated from sibships with a higher infection success also reached a larger size in the copepod: r_s = 0.74, N = 8, P = 0.0366 (Figure 2a).

Table V-2: Genetic correlations for traits within and among hosts for full-sib families (parasite sibships). r^2 from the family mean correlation is shown in square brackets as an estimate of the proportion of genetic variance attributable to effects of the same or linked genetic factors. * $P < 0.05$.

	hatching	infectivity in copepod	size in copepod	infectivity in fish	weight in fish	RLU in fish	hepatosomatic index
hatching	1	.04 [0%]	.11 [1%]	-.86 * [74%]	.59 * [35%]	-.05 [0%]	-.10 [1%]
infectivity in copepod		1	.74 * [54%]	.20 [4%]	.18 [3%]	-.95 * [90%]	. 81 * [66%]
size in copepod			1	.04 [0%]	.39 [15%]	-.71 * [51%]	.43 [18%]
infectivity in fish				1	-.79 * [62%]	-.12 [1%]	.12 [1%]
weight in fish					1	0 [0%]	-.14 [2%]
RLU in fish						1	-.88 * [78%]
hepatosomatic index							1

Fitness of tapeworms in their second intermediate host, the stickleback

Of the 176 fish that had been exposed to infected copepods, 14 (7.9%) died before the end of the experiment and 2 fish were excluded from parts of the analyses because they suffered from another illness (see methods for detail). Mortality of fish was independent of the parasite sibship (LR $\chi_7^2 = 5.83$, $P = 0.56$). In total, 33.95% of the fish were infected. The likelihood of infection was not significantly explained by any of the following factors included into the model: parasite sibship (LR $\chi_7^2 = 2.37$, P = 0.94), fish family (LR $\chi_3^2 = 6.72$, d.f. = 3, $P = 0.08$), fish gender (LR $\chi_1^2 = 0.30$, P = 0.59), and fish condition factor before infection (LR $\chi_1^2 = 0.76$, P = 0.38). Size of parasite (N=21) was not significantly influenced by any of the effect variables in the model: parasite sibship ($F_{6, 9} = 1.31$, $P = 0.34$), fish family ($F_{3, 9} = 2.23$, $P = 0.15$), fish gender ($F_{1, 9} = 3.02$, $P = 0.17$) or weight pre infection ($F_{1, 9} = 0.42$, $P = 0.54$). In contrast to the first intermediate host, where a positive correlation between the

infectivity and the mean procercoid size of the tapeworm sibships had been found, we observed a negative correlation between infectivity and mean tapeworm (i.e. plerocercoid) size in the fish host (r_s = - 0.79, N = 7, P = 0.0362).

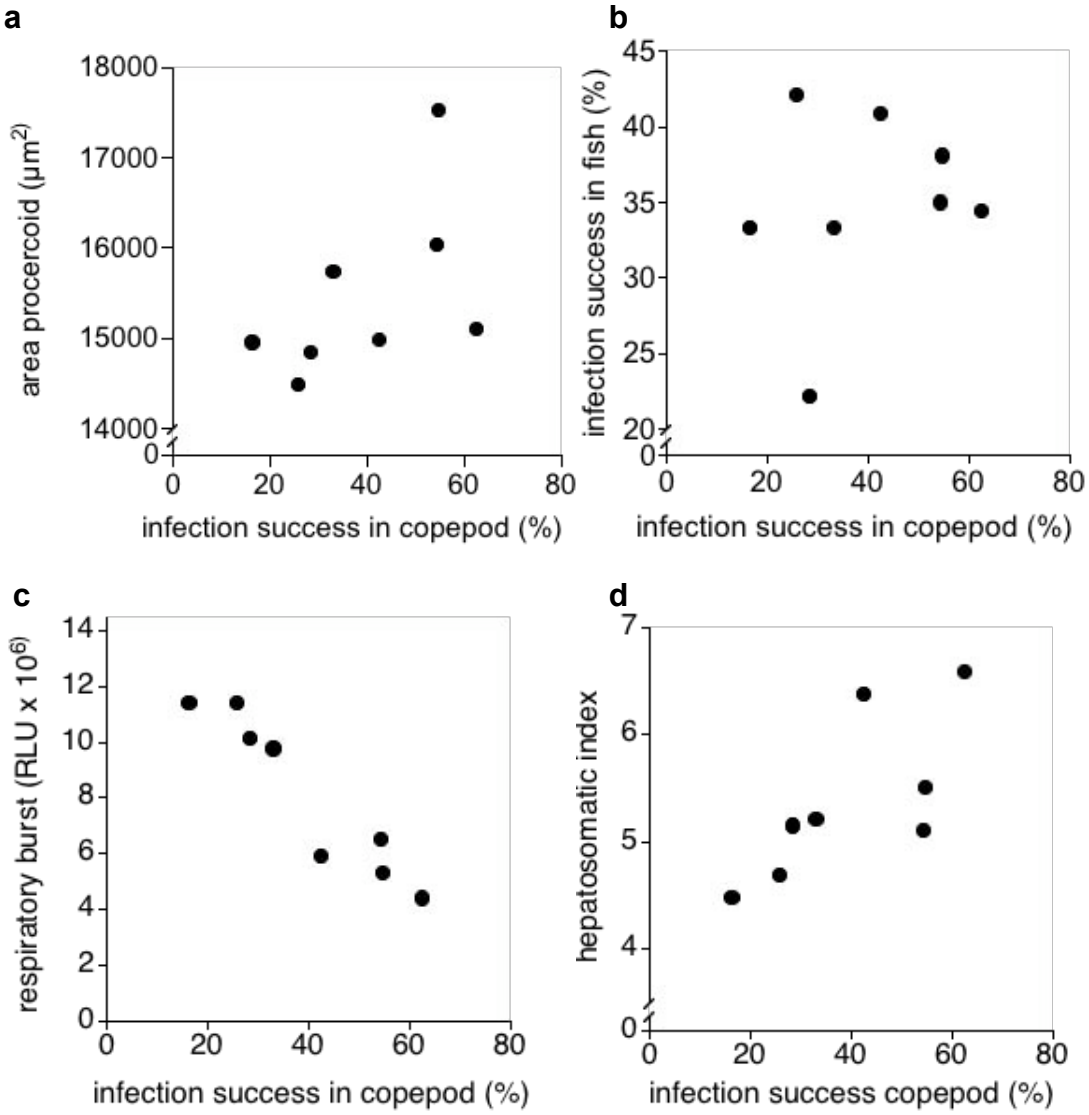


Figure V-2: Relation between infectivity (%) of sibships of the tapeworm *S. solidus* in its first intermediate host (the copepod *M. albidus*) and (a) worm size 12 d.p.i. in the copepod, (b) infectivity in the second intermediate host (the stickleback fish *G. aculeatus*), (c) stickleback innate immune response (respiratory burst 7 d.p.i.), and (d) stickleback body condition (hepatosomatic index 7 d.p.i.). Each data point shows the mean of a parasite sibship, i.e. 22–54 individuals in the copepod host, and 2–6 individuals in the fish host, respectively.

Trade-offs in fitness between the different developmental stages of the parasite

Parasite sibships varied in hatching success from egg clutches (LR χ_7^2 = 43.31, P < 0.0002). We may thus expect that sibships, which did not hatch well, might compensate for this disadvantage later during their life, i.e. by being better in infecting the first or second intermediate host. We found such a relationship with regard to infection of the fish host (r_s = - 0.86, N = 8, P = 0.0061), but not in the copepod host (r_s = 0.04, N = 8, P = 0.93).

We expected to find a trade-off between the infection success of parasite genotypes in the copepod and in the fish host. However, among worm sibships there was no negative correlation between these traits (r_s = 0.20, N= 8, P = 0.63) (Figure 2b). Another factor that could have influenced infectivity in the fish is the size of the procercoid; bigger parasites could potentially have an advantage when infecting a fish. We could not show that in this experiment: r_s = 0.04, N = 8, P = 0.93. Furthermore, there was no significant relation between mean procercoid size (in the copepod) and mean plerocercoid weight (in the fish) among parasite sibships (r_s = 0.39, N = 7, P = 0.38).

Parasite virulence and host immunity

Tapeworm sibships varied in the intensity of activation of their fish hosts' innate immune response, as estimated from the respiratory burst reaction. Since previous work has shown that the impact of *S. solidus* on the fish immune system could be assessed best with the respiratory burst reaction (Kurtz et al. 2004), we concentrated on results from this assay in further analyses. This measure correlated with another measure for the activity of the innate immune system, the G: L ratio ($F_{1, 151}$ = 22.97, P< 0.0001, r = 0.36), i.e. the proportion of granulocytes in relation to lymphocytes. Moreover, the use of the G: L ratio as an innate immune measure instead of the respiratory burst would lead to qualitatively similar conclusions, although effects are more pronounced with respiratory burst as measure.

Sibships better at infecting the copepods also elicited a weaker innate immune reaction (respiratory burst) 7 days post infection (p.i.) in the stickleback (r_s = -0.95, N = 8, P = 0.0003) (Figure 2c). Moreover, fish infected with these genotypes were in better condition as estimated from the hepatosomatic index (r_s = 0.81, N = 8, P = 0.0149) (Figure 2d). However, 35 days p.i., the correlation between infectivity in the

copepod and fish immune stimulation disappeared (r_s = 0.21, N = 7, P = 0.64), while the correlation with the hepatosomatic index remained as a trend (r_s = 0.75, N = 7, P = 0.0522). This was not due to a positive correlation between plerocercoid weight and hepatosomatic index (r_s = - 0.143, N = 7, P = 0.76), showing that fish were not in worse condition in consequence of infection with parasites that may exploit them more.

Discussion

Sibships of the tapeworm *S. solidus* varied considerably in fitness parameters in the first and the second intermediate host, copepods and sticklebacks. Analysis of heritability indicates that such variation was partly genetic, especially for traits measured in the copepod. It should be noted, however, that a full-sib design does not control for common environmental, dominance, and maternal effects. For parasite traits in the fish intermediate host, most heritability estimates were not significantly larger than zero. This might be caused by the lower sample sizes of parasites in the fish, but could also be a consequence of strong phenotypic variation in parasite traits that is caused by the fish host. This interpretation is corroborated by previous studies showing that phenotypic and genetic factors of the stickleback influence growth of *S. solidus* (Barber 2005; Kurtz et al. 2004).

Contrary to our initial expectation, we did not find that parasite sibships, which performed weakly in the copepod host, were superior in the fish host. This absence of a trade-off, combined with the indication that parasite performance is partly based on genetic variation, raises the question as to what might maintain apparently inferior genotypes in the population. One possible explanation is the above-mentioned lack of heritability of traits measured in the fish host. Another possibility is that trade-offs might occur with parasite performance in the definitive, bird host, rather than the second intermediate host. This interpretation is especially intriguing since previous studies found trade-offs in fitness between intermediate and definitive hosts (Davies et al. 2001; Gower & Webster 2004). However, we consider this interpretation unlikely in our particular system, where it is fairly safe to assume that the definitive host is rather unimportant for parasite fitness. First, *S. solidus* appears to cause little harm to the bird (Clarke 1954; Tierney & Crompton 1992), which leads to the

assumption that there is no relevant selection against *S. solidus* in the definitive host. Second, and most importantly, the worms do not grow any more in the bird (Clarke 1954). They seem to entirely rely on the resources obtained from the intermediate hosts for the short phase of reproduction in the birds' gut (Tierney & Crompton 1992; Schärer & Wedekind 1999). This implies that worm size achieved in the intermediate hosts determines establishment and egg production in the definitive host (Tierney & Crompton 1992). Therefore, the relevance of the two intermediate hosts for parasite fitness in this system is comparable to the intermediate and definitive hosts in other systems.

The arguably most important reason for the absence of a trade-off in infectivity between the two intermediate hosts is revealed by a closer look at the interaction of the tapeworms with their hosts' immune systems. We here propose that worm sibships that are well adapted to components of the innate immune system will benefit in both copepod and stickleback hosts. This proposition is not unrealistic, since invertebrate and vertebrate innate immunological receptors recognize similar pathogen-associated molecular patterns, such as peptidoglycans (Guan et al. 2004; Janeway 1999; Janeway & Medzhitov 2002; Salzet 2001). Worms that remain undetected by the copepod immune system may thus represent worms that are well adapted to evade innate immune systems in general. However, such worms might have a disadvantage when interacting with fish adaptive immunity that will normally displace innate immunity later during an infection. This hypothesis is supported by our observations that (1) tapeworm sibships, which are highly infective to the copepod, elicit a weaker innate immune response, as estimated from the respiratory burst reaction, at an early stage of fish infection (7 days p.i.); (2) this relation disappeared at a later stage of the infection (35 days p.i.); (3) there was a negative correlation between the infectivity of a sibship (that seems dominated by innate defence) and its mean plerocercoid size 35 days p.i. (that might be influenced by adaptive immunity). Unfortunately, we do not have any direct measures of adaptive immunity in the current experiment. However, we have reason to believe that adaptive immunity influences worm size in the stickleback, since size (but not the likelihood of infection *per se*) has previously been shown to depend on stickleback MHC genetics, which are central to the adaptive immune system (Kurtz et al. 2004). Taken together, these results indicate that parasites with complex life cycles could

face a dilemma when adapting to different components of their hosts' immune systems. Since each individual parasite has a limited pool of resources to invest, it might have to allocate its resources strategically (e.g. into diverse immune evasive strategies).

Does this have any consequences for the virulence of the different parasite genotypes? We found that worm sibships that are highly infective to copepods also were in fish with better body condition, as estimated from the hepatosomatic index. In sticklebacks, this measure of energy reserves is a meaningful estimate of the negative consequences of tapeworm infection (Kurtz et al. 2004). The severe effects on fish condition may be a direct, immunopathological consequence of the activated innate immune system, as has been found in other systems (see Lochmiller & Deerenberg 2000). Importantly, the harmful effects of a parasite on its host might be a consequence of deficient adaptation of the parasite to parts of its host's immune system rather than a direct effect resulting from resource drain to the parasite. This seems to be the case in our system, since the more harmful parasites for the stickleback did not grow bigger. Thus, parasites may not necessarily benefit from such harmful effects, i.e. virulence and fitness of a parasite might be uncoupled. What could then maintain the more virulent genotypes in the population? One possibility, yet unstudied here, is transmission to the definitive host, which might be enhanced in fish that suffer more from the infection (Giles 1983; Lester 1971).

What are the population consequences of the above findings? Under natural situations, most sticklebacks will be infected with worm sibships that are highly infective to the copepod. At the same time, these genotypes were also relatively benign for the fish. As a side effect, infection of the invertebrate host thus represents a 'filter' that reduces infection of the subsequent, vertebrate host with the most harmful parasites. These findings might also give an explanation for observations from serial passage experiments, where the exclusion of a first, intermediate host from a parasite's life cycle, through artificial transfer of parasites among individuals of the second host alone, lead to increased virulence in that host (Ebert 1998; Mackinnon & Read 2004).

In conclusion, if different genotypes of parasites in multi-host life cycles are adapted to cope either with parts of the invertebrate or the vertebrate immune

systems, this could explain performance and virulence of parasites in this and potentially other multi-host parasites.

Conclusion

Before I started working on *Schistocephalus solidus* as a model system for a parasite with a complex life cycle, I did not assume that the host parasite interactions would be straightforward. Three years later I am still surprised by how complicated complex life cycles really are, and how little we actually know about them. Host parasite interactions in complex life cycles have, only recently, started to become a focus of research in evolutionary parasitology (Davies et al. 2001; Gandon 2004; Gower & Webster 2004; Parker et al. 2003a, b). Previously, theoretical and empirical work mainly focused on single-host systems (Frank 1996; Galvani 2003; Little & Ebert 2001), which is surprising because the majority of parasites possess a complex life cycle.

The results presented in this thesis underline that host parasite interactions depend on several steps of defence and attack that determine the success of parasite infection and host resistance. From the parasite's point of view, it is not only sufficient to successfully cope with the host's immune system, but it also has to reach (chapter I & II), enter and become established in the host (chapter III), before finally evading the host immune defence (chapter IV). To complicate things further, parasites in complex life cycles have to perform each of these steps for every host anew. Thus, parasites are probably not perfectly adapted to each environment (host), due to limited resources to equally invest in all traits or genetic constraints between traits (chapter V).

For more than 80 years scientists have been fascinated by parasites that potentially manipulate their hosts. Research mainly focused on whether behavioural manipulation of hosts is a direct adaptive effect, or merely a by-product of infection (Milinski 1990; Poulin 1994a). Recently, several behavioural parasitologists commented on this ongoing debate and suggested to not only try to differentiate between the two possibilities, but understand hosts as 'deeply modified organisms' (Cézilly & Perrot-Minnot 2005; Thomas et al. 2005; Webster 2005). The data from chapters I and II support this view: *S. solidus* infected copepods do not only change in activity, but also in storage of carotenoids during the course of the infection. To fully understand behavioural changes of infected hosts, more knowledge about mechanisms and potential costs of manipulation is needed (Thomas et al. 2005). However, the physiology of host parasite interactions is difficult to disentangle, especially concerning the complex interaction between immunity and the nervous

system (Thomas et al. 2005). When parasites invade host tissue, they trigger complex immune responses, which often lead to a release of neuroactive compounds influencing brain and behaviour of infected organisms (Bechter 2001; Dantzer 1999). Thus, changes in host behaviour may be an unavoidable result of parasite-induced immune response or immuno-suppression (Kavaliers et al. 1999).

Interestingly, this could be also the case in *S. solidus* where indications for suppression of the stickleback immune system during infection were found (Scharsack et al. 2004). Potential candidates involved in immunosuppression or immune-avoidance are carbohydrates on parasite surfaces (Ma et al. 2002; Plows et al. 2005; Tomaska & Parish 1981). In this thesis I provided evidence that surface carbohydrate composition of *S. solidus*, in the two intermediate hosts, varies between the two parasite stages (pro- and plerocercoid) and that individual variation in surface carbohydrate composition can be directly linked to parasite fitness in the fish host (Hammerschmidt & Kurtz 2005b; chapter IV). This study was the first one that linked individual variation in surface characteristics of a parasite to its fitness, and thus combined parasitological with evolutionary ideas in a multidisciplinary concept. To fully understand diseases caused by parasites, and thus being able to find suitable vaccines or drugs, it is necessary to include evolutionary concepts in medical parasitology.

Carbohydrates on parasite surfaces could also be involved in protecting underlying tissues of parasites from enzymatic digestion in the intestines of their vertebrate hosts (Hammerschmidt & Kurtz 2005b; chapter IV; Ingold et al. 2000; Obregón-Henao et al. 2003; Sandeman & Williams 1984). The phase between ingestion by the host and reaching the target tissues is considered to be highly relevant in many parasite species in determining infection success (Barker 1970; Gemmel 1964). Analysing the infestation process of *S. solidus* showed that a successful infection does not only depend on evading the host's immune system, but also on surviving in the host's intestinal tract and penetrating the mucosal wall (chapter III).

The adaptation to different host physiologies, especially the immune system, strongly influences the evolution of parasite virulence in a multi-host parasite. I found *S. solidus* tapeworms to trade-off adaptation towards different parts of the hosts' immune systems (Hammerschmidt & Kurtz 2005a; chapter V). Moreover, parasite sibships that performed better in the invertebrate host were less virulent in the fish. This result generally stresses the role of a first intermediate host or vector (which is

often an invertebrate), for shaping virulence in the next host, which is mostly a vertebrate. Consequently, on a population level, if most of the vertebrate hosts are infected with parasites that are highly infective to the invertebrate (infectivity in the vertebrate is equal for all sibships), they are at the same time less virulent in the vertebrate. The invertebrate host might therefore represent a 'filter' that reduces infection of the subsequent vertebrate host with the most harmful parasites. Similar evidence comes from serial passage experiments in which generally, after exclusion of the first intermediate host from the cycle, virulence increased in the second intermediate host (Ebert 1998; Mackinnon & Read 2004). These results suggest caution when manipulating the population of invertebrate hosts or vectors to control vector-born diseases like malaria because consequences for the vertebrate hosts, e.g. humans, are poorly understood. Although more empirical and theoretical studies are definitively needed, this thesis contributed to a better understanding of host parasite interactions in complex parasitic life cycles.

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CURRICULUM VITAE

Katrin Hammerschmidt

geboren am 19. März 1977
in Paderborn

AUSBILDUNG

seit Dez. 2002	Promotion in der Arbeitsgruppe Evolutionsökologie am Max Planck Institut für Limnologie in Plön
Sept. 2002	Abschluss des Biologie Studiums mit der Note ‚sehr gut'
2001 - 2002	Stipendium der Heinrich Hertz-Stiftung: Diplomarbeit bei der ‚Marine Biological Association of the United Kingdom' (MBA) in Plymouth, **Thema:** ‚Reproductive strategies in the colonial simultaneously hermaphroditic protochordate *Diplosoma listerianum*', Bewertung: ‚sehr gut'
Mai 1999	Diplom-Biologen-Vorprüfung, Note: ;sehr gut'
1996 - 2002	Studium der Biologie mit Studienziel Diplom an der Westfälischen Wilhelms-Universität Münster (WWU)
1993	Viermonatiger Besuch der Goethe-Schule in Buenos Aires
1987 - 1996	Städtisches Gymnasium Marsberg; Erwerb der ‚Allgemeinen Hochschulreife' mit der Note 2,1

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Erklärung

Hiermit versichere ich, dass diese Abhandlung – abgesehen von der Beratung durch meine akademischen Lehrer- nach Inhalt und Form meine eigene Arbeit ist und dass ich keine anderen als die angegebenen Hilfsmittel und Quellen verwendet habe. Die Arbeit hat bisher weder ganz noch zum Teil an anderer Stelle im Rahmen eines Prüfungsverfahrens vorgelegen.

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Katrin Hammerschmidt