Host specificity and adaptation of *Schistocephalus* to its stickleback hosts

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Summary

Parasites are a major evolutionary driving force. They impose a selection factor not only on individual hosts, but also on whole populations altering natural and sexual selection. The coevolution between hosts and parasites leads to an evolutionary arms race, where hosts evolve towards higher resistance and parasites towards increased exploitation. Parasites have evolved many different adaptations to increase their potential to successfully infect and exploit their hosts. A parasite with a multi-host life cycle may use the different hosts for different purposes and must adapt to different conditions at each stage. All adaptations should ultimately increase the parasite's fitness: through higher growth and higher rates of transmission and reproduction.

The work of my thesis examines the adaptations of parasites, specifically tapeworms of the genus *Schistocephalus* (*S. solidus* and *S. pungitii*), to their host organisms: three-spined sticklebacks (*Gasterosteus aculeatus*) and nine-spined sticklebacks (*Pungitius pungitius*). *Schistocephalus* has long been known to be very specific regarding the second intermediate (stickleback) host, which indicates close coevolution of these species. In chapter I of this thesis I examined this high degree of host specificity by experimentally exposing sticklebacks to either their specific or the incompatible parasite species and monitoring the infection process histologically. This could show that the incompatible parasite species can still establish in the stickleback, but is eliminated within the first two weeks after infection. I also tested, whether the known immune manipulation by *S. solidus* in three-spined sticklebacks allows a superinfection with the incompatible parasite in sequential exposures, but the results indicate that this is not possible.

In the second experiment I hybridized two different *Schistocephalus* species (*S. solidus* and *S. pungitii*) in an *in vitro* breeding system and measured fitness relevant traits throughout the whole life cycle. I could show that the two species are capable of producing viable hybrid offspring, even though the outcrossing and hatching rates are lower in these pairings than in the parental species. Nevertheless, the hybrids exhibit no decreased infection rate in the first and second intermediate hosts and surprisingly show an extended host range, as they are able to infect both stickleback species, while the parental lines can only infect their specific host.

This is surprising, as natural hybrids between *S. solidus* and *S. pungitii* have not yet been observed and molecular data indicates a deep lineage divergence and no gene flow. In the

next part of this thesis I therefore tested, if prezygotic barriers prevent hybridization in natural populations. The results suggest that the species can hybridize in natural hosts, there are no barriers to hybridization in sympatric populations and the parasites even prefer parasites of the different species over conspecifics in a mate choice experiment.

In summary, these results indicate that host specificity in *Schistocephalus* is presumably maintained in this system due to the specific reaction of the stickleback's immune system, even though the advantages and the mechanisms are still unclear. It is possible that the high degree of host specificity is important for successful long term interactions with the stickleback immune system, even though our results indicate no trade-off at this level.

The ability of a parasite to successfully establish and exploit a host is also determined by parasite virulence, which depends on many factors that also include intraspecific interactions among co-infecting parasites. In the last part of this thesis, I investigated the plasticity of individual parasite virulence using experimental co-infections with two different strains of *S. solidus* that differ in virulence within three-spined sticklebacks. This showed that intraspecific interactions alter individual virulence in *S. solidus*, where the less virulent parasite benefits from the presence of a high-virulent conspecific and the high-virulent parasite exhibits reduced virulence in heterologous co-infections.

This thesis demonstrates that these parasites use numerous and elaborate approaches to adapt to their host. Furthermore, the outcome of a parasitic infection is dependent on the close coevolution between parasitic exploitation strategies and host defenses, and finally, these interactions become even more complex with multiple infections.

Zusammenfassung

Parasiten sind eine bedeutende Triebkraft in der Evolution. Sie üben nicht nur einen großer Selektionsdruck auf einzelne Wirte aus, sondern auch auf ganze Wirtspopulationen, indem sie die natürliche und sexuelle Selektion beeinflussen. Die Koevolution zwischen Wirten und ihren Parasiten führt zu einem evolutionären Wettrüsten, bei dem sich Wirte mit höheren Resistenzen und Parasiten mit besseren Ausbeutungsstrategien anpassen. Parasiten haben viele verschiedene Anpassungen entwickelt, um ihr Potenzial zur erfolgreichen Infektion und Ausbeutung ihrer Wirte zu erhöhen. Parasiten, deren Lebenszyklus mehrere verschiedene Wirte beinhaltet, welche verschiedenen Zwecken dienen, müssen sich an die unterschiedlichen Bedingungen in den Wirten auf jeder Stufe anpassen. Alle Anpassungen sollten letztendlich die Fitness der Parasiten erhöhen: durch höhere Wachstums-, Transmissions- und Reproduktionsraten.

Meine Dissertation untersucht die Anpassungen von Parasiten, insbesondere von Bandwürmern der Gattung *Schistocephalus* (*S. solidus* und *S. pungitii*), an ihre Wirtsorganismen: Dreistachlige Stichlinge (*Gasterosteus aculeatus*) und Neunstachlige Stichlinge (*Pungitius pungitius*). Es ist seit langem bekannt, dass *Schistocephalus* sehr spezifisch bei der Wahl seines zweiten Zwischenwirts (in diesem Fall Stichlinge) ist, was eine enge Koevolution dieser Arten anzeigt. Im ersten Kapitel dieser Arbeit habe ich diese hohe Wirtsspezifität durch experimentelle Infektionen von Stichlingen mit ihren spezifischen oder inkompatiblen Parasiten und eine histologische Verfolgung des Infektionsprozesses untersucht. Die Ergebnisse zeigen, dass die inkompatiblen Parasiten zwar die Körperhöhle des Stichlings erreichen, dort allerdings innerhalb der ersten zwei Wochen nach Infektion durch das Immunsystem des Stichlings beseitigt werden können. Weiterhin habe ich getestet, ob die bekannte Immunmanipulation von *S. solidus* in Dreistachligen Stichlingen eine Superinfektion mit einem inkompatiblen Parasiten in simultanen oder sequentiellen Infektionen mit beiden Parasiten ermöglicht. Die Ergebnisse zeigen, dass dies nicht der Fall ist.

Im zweiten Kapitel dieser Dissertation habe ich die beiden *Schistocephalus* Arten *S. solidus* und *S. pungtii* in einem *in vitro* System hybridisiert und fitnessrelevante Merkmale im gesamten Lebenszyklus der Parasiten gemessen. Ich konnte damit zeigen, dass die beiden Arten lebensfähige Hybrid-Nachkommen erzeugen können, auch wenn die Auskrezungs- und Schlupfraten in diesen Kombinationen niedriger sind als bei den reinen Elternlinien. Dennoch

zeigen die Hybride keine verminderten Infektionsraten bei beiden Zwischenwirten (Copepoden und Fischen) und zeigen erstaunlicherweise ein erweitertes Wirtsspektrum auf der Ebene des zweiten Zwischenwirts. Sie sind in der Lage, beide Stichlingsarten zu infizieren, während die Elternlinien nur ihren spezifischen Fischwirt infizieren können.

Dieses Ergebnis überrascht, da natürliche Hybride zwischen *S. solidus* und *S. pungitii* bisher nicht beobachtet wurden und molekulare Untersuchungen eine relativ große genetische Divergenz zwischen den beiden Arten und keinen Genfluss andeuten. Im nächsten Kapitel dieser Dissertation habe ich daher untersucht, ob präzygotische Barrieren die Hybridisierung in natürlichen Populationen der Parasiten verhindern. Die Ergebnisse zeigen, dass die beiden Arten in einem natürlichen Endwirt hybridisieren können, es keine Barrieren für Hybridisierung in sympatrischen Populationen der Parasiten gibt, und dass die Parasiten in einem Partnerwahlversuch Parasiten der anderen Art gegenüber Artgenossen bevorzugen. Zusammenfassend zeigen diese Ergebnisse, dass die Wirtsspezifität im *Schistocephalus*-Stichlings-System womöglich durch die spezifische Reaktion des Fischimmunsystems aufrecht erhalten wird, obwohl die Vorteile und Mechanismen dafür noch unklar sind. Es ist möglich, dass der hohe Grad der Spezifität wichtig für eine erfolgreiche Interaktion mit dem Stichlingsimmunsystem auf lange Zeit ist, auch wenn unsere Ergebnisse keinen Beeinträchtigungen auf dieser Ebene zeigen.

Die Fähigkeit eines Parasiten sich erfolgreich in einem Wirt zu etablieren und diesen auszunutzen hängt auch von dessen Virulenz ab, die von vielen verschiedenen Faktoren beeinflusst wird, unter anderem auch von den Interaktionen zwischen koinfizierenden Parasiten. Im letzten Teil meiner Dissertation habe ich daher die Plastizität der Virulenz eines einzelnen Parasiten in experimentellen Koninfektionen von Dreistachligen Stichlingen untersucht. Dazu habe ich verschiedenen Stämme von *S. solidus* verwendet, die sich in ihrer Virulenz unterscheiden. Diese Ergebnisse zeigen, dass die intraspezifischen Interaktionen zwischen den Parasiten die Virulenz eines einzelnen Parasiten stark beeinflussen: Der weniger virulente Parasit zieht Vorteile aus der Anwesenheit eines hoch virulenten Parasiten, während der hoch virulente Parasit in heterologen Koinfektionen eine verminderte Virulenz zeigt.

Die Dissertation zeigt, dass Parasiten zahlreiche und sehr aufwendige Strategien nutzen, um sich an ihre Wirte anzupassen. Außerdem ist das Ergebnis einer parasitären Infektion auch von der engen Koevolution von parasitischen Ausbeutungsstrategien und spezifischen Abwehrmechanismen der Wirte abhängig, und schlussendlich werden diese Interaktionen noch komplexer in Infektionen mit mehreren Parasiten.

Introduction

Host-parasite coevolution

Although the definition of parasitism varies, Price resorted to a definition from Webster's Third International Dictionary (Gove, 1964) in his book Evolutionary Biology of Parasites (1980):

"A parasite is an organism living in or on another living organism, obtaining from it part or all of its organic nutrient, commonly exhibiting some degree of adaptive structural modification, and causing some degree of real damage to its host."

Because of the great biological diversity and different ways to exploit hosts, this definition includes many plants and animals. Even though there is some debate regarding the most appropriate definition for parasitism, it is likely that all organisms encounter parasites at some point in their life (Poulin, 1996). As a testament to their enormous biological diversity, it has been claimed that parasitism is the most common way of life on earth and that there are more parasitic than non-parasitic species (Kuris *et al*., 2008; Windsor, 1998).

Coevolution can arise where two or more organisms, such as hosts and parasites or plants and pollinators, exert selection pressure on and evolve in response to the other species. While plants and pollinators experience mutualistic coevolution, hosts and parasites undergo antagonistic coevolution. Both hosts and parasites aim to maximize their reproductive fitness. Parasites evolve to maximize their fitness through host exploitation, whereas hosts evolve defense mechanisms to reduce damage caused by parasites. This arms race has been described in the Red Queen hypothesis (Van Valen, 1973), where the analogy is taking “all the running you can do, to keep in the same place” (Lewis Carroll, Alice Through the Looking Glass, 1872). When one species evolves e.g. new alleles that give an advantage in infection, the other species is adversely affected by this and has to evolve e.g. more elaborate defense mechanisms to keep up, which is explained in this metaphor. This arms race is often not unidirectional (Lively, 1996), but can lead to a fluctuation of parasite and host genotypes. A parasite genotype that is successful in host infection may rise in frequency, therefore imposing a considerable selection factor on the host population. Host genotypes that are resistant against this parasite genotype have a selective advantage and become more frequent, which leads to a decrease in the parasite genotype, allowing a new parasite genotype to take over and restart the cycle. Alternatively, population size fluctuations and stochasticity may

request different dynamics to explain host-parasite coevolution, as suggested by Gokhale and colleagues (2013), where recurrent selective sweeps replace the red queen dynamics mentioned above.

Whichever theory applies, both hosts and parasites are constantly co-adapting to each other, a process driven by selection factors that increase both host and parasite fitness. For parasites, this often means a higher rate of transmission and an increased chance of persistence in the host. Parasite fitness tends to be improved by, in a broad sense, two strategies. The first being manipulation of the host immune system whereby the immune system either doesn't recognize the parasite or doesn't mount a response to the parasite; whereas, the second is the manipulation of the host's behavior. Through a wide variety of behavioral manipulations, parasites can force the host to otherwise "unnatural" behaviors that increase the parasite's fitness through a higher transmission rate or increased reproduction (Moore, 2002). It is also worth noting that most parasites have much shorter generation times than their hosts, which is advantageous to parasites in the co-evolutionary arms race (Hamilton, 1980; Lively, 1999).

To improve their fitness hosts have strategies that can also be grouped into two broad groups. These are resistance, reduction of infection probability, and tolerance, limitation of harm caused by parasitic infection (Råberg *et al*., 2009). Mechanisms involved in host defense can include behavioral avoidance, structural defense and immune defense (innate or adaptive). The host defense can be increased through physical and chemical barriers, behavioral avoidance, changes in the innate and adaptive immune response, and through trans-generational immune priming.

This tightly linked coevolution can also influence traits outside the "attack" and "defend" cycle. For example, is has been known that parasites can alter sexual selection in a species, where parasitic infections influence sexually selected traits, such as breeding colorations leading females to choose males with a low parasite load over highly infected individuals (Andersson, 1994; Hamilton & Zuk, 1982).

Parasites can therefore impose a big selection factor on a population. It is now widely accepted that parasites play a role in the evolution of sex (Hamilton *et al*., 1990; Hamilton, 1980; Maynard-Smith, 1978) and the origin of species diversity (Haldane, 1949; Hutchinson, 1959; Summers & McKeon, 2003) by imposing natural and sexual selection factors on a population.

Parasite adaptations to the host

Parasites have evolved many different adaptations that allow them to successfully exploit a host and maximize their transmission rate and offspring. In many parasite taxa these adaptations span complex life cycles that include several hosts at different life stages. The evolution of complex life cycles, by the addition of a new host to the life cycle, is adaptive if this leads to an increase in transmission or reproductive output of the parasite (Combes, 1995; Ewald, 1995; Morand, 1996). Ewald (1995) argues that the benefits associated with specialization on different hosts for different resources (food, transport) led to the increase in complexity in parasite life cycles. However, the ability of the parasite to efficiently exploit different resources throughout its ontogeny (Ebenman, 1992) seems to be a prerequisite for this hypothesis. Consequently, parasites that use different hosts throughout their life cycle have to adapt to changing conditions in each host.

A parasite's level of host specificity, at any particular stage of the life cycle, can vary. This specificity is defined as the number of hosts a parasite at a given stage can successfully infect and exploit. It ranges from high in generalist parasites to very low in highly specialist parasites. This highly important parasite characteristic is determined by an "encounter filter model" (Combes, 1991). This filter consists of an actual encounter filter that is determined by which hosts the parasites encounter and a compatibility filter that is determined by physiological factors that allow a parasite to infect a given host. Only if both filters overlap, can a parasite successfully become established in the host. In complex life cycles, specificity may also depend on the function of the host at a specific stage. If hosts mainly serve as transportation vehicles, a lower specificity may be advantageous, whereas host stages that are severely exploited for nutrients require a closer adaptation, which can favor higher specialization by the parasite. The degree of specialization may also be associated with the virulence of a parasite, as it has been suggested that more generalist parasites tend to be less virulent (Garamszegi, 2006).

Virulence is defined as damage caused by the parasite, leading to a reduction of fitness in the host (Ebert & Herre, 1996; Ewald, 1995b). Virulence can either be a by-product of host exploitation or adaptive, if fitness reduction directly increase parasite transmission (e.g. if the host is more susceptible to predation, which would allow the parasite to get transmitted to the next host). Natural selection will not necessarily favor evolution towards higher virulence, but rather that level of virulence that is associated with the highest parasite fitness (Ewald, 1993), i.e. optimal virulence. In sympatric populations of hosts and parasites, a parasite can

reach a level of optimal virulence through close coevolution with their corresponding hosts (Galvani, 2003). A parasite exploits its host to a level that maximizes parasite fitness, whereas mismatches between hosts and parasites could lead to over- or underexploitation of the host, both with negative fitness consequences for the parasite.

Traits that increase parasite fitness on one host but decrease fitness on other hosts can favor evolution towards a higher degree of host specificity. Assuming these hosts are sufficiently abundant, the benefits of specialization might outweigh the cost of a decreased number of potential hosts a parasite can infect (Jaenike, 1990). It is now widely accepted that host specificity plays a key role in parasite speciation (Brooks & McLennan, 1993; Shaw, 1994; Thompson, 1994).

All adaptations of parasites to their host also depend on other co-infecting parasite species. As naturally infected hosts harbor usually numerous individuals of several different parasite species, the effects of intraspecific and interspecific parasite-parasite interactions may be underestimated in experimental studies of host-parasite coevolution (Milinski, 2014).

In this thesis I investigated the host specificity and adaptations of *Schistocephalus* spp. to their stickleback hosts. This system allows investigating a wide range of traits throughout the parasite life cycle and their consequences for the fish host, which determine both parasite and host fitness.

Model system

Sticklebacks

Three-spined sticklebacks (*Gasterosteus aculeatus*) and nine-spined sticklebacks (*Pungitius pungitius*) both are members of the family Gasterosteidae, which inhabit marine or freshwater environments in the temperate and subarctic regions of the Northern Hemisphere (Nelson, 1994). After the last glaciation event, sticklebacks have repeatedly colonized lakes and streams which led to rapid adaptive radiation (Bell & Foster, 1994; Wootton, 1976) that include a number of morphological traits.

Both fish species (Figure 1) share similar habitats, although nine-spined sticklebacks may prefer niches with more dense vegetation, whereas three-spined sticklebacks may dominate in more open parts of the water body, also supplying different food sources (Coad & Power, 1973a, 1973b). Nevertheless, both niches and diet of the two stickleback species overlap in many regions (Hynes, 1950; Zander *et al*., 1984).

Figure 1: Two members of the Gasterosteidae, three-spined stickleback (*Gasterosteus aculeatus*, picture above) and nine-spined stickleback (*Pungitius pungitius*, picture below). Pictures kindly provided by Sophie Bodenstein (three-spined stickleback) and Kenyon Mobley (nine-spined stickleback).

The three-spined stickleback has become an important model organism in evolutionary biology, evolutionary ecology, parasitology and behavioral studies (Barber & Nettleship, 2010; Barber, 2013; Gibson, 2005; Huntingford & Ruiz-Gomez, 2009). In contrast to many other model organisms, much is known about its natural history, ecology and evolutionary biology (Bell & Foster, 1994; Wootton, 1976). More recently, since its genome has been published, the development of genetic and genomic tools increased and opened up new possibilities in stickleback research (Gibson, 2005; Kingsley *et al*., 2004). These include the genetics underlying morphological variation (Chan *et al*., 2010; Colosimo & Hosemann, 2005; Kingsley & Peichel, 2007; Miller *et al*., 2007; Shapiro *et al*., 2004) and the genomic basis of adaptive evolution (Jones *et al*., 2012). Similar prerequisites in nine-spined sticklebacks led to their emergence as a model system (Merilä, 2013), allowing comparative studies between two closely related species in evolutionary biology and parasitology.

Another recent field in which sticklebacks have proven to be important model organisms is the study of local adaptation in host-parasite interactions (Kalbe & Kurtz, 2006; Matthews *et al*., 2010; Scharsack *et al*., 2007) and patterns of parasite-driven local genetic adaptation (Eizaguirre *et al*., 2012; Konijnendijk *et al*., 2013; Nuismer & Gandon, 2008). This is facilitated by a large number of parasites from various taxa and the possibility to handle sticklebacks and several naturally occurring parasites in the laboratory, allowing experimental infection studies. Furthermore, several tools allow accessing the innate and adaptive immunity of sticklebacks with which the consequences of parasitic infections can be measured.

Part of the adaptive immune system in jawed vertebrates is the major histocompatibility complex (MHC) which plays a crucial role in the presentation of antigens and has also been known to be important for resistance against parasites (Kurtz *et al*., 2004; Wegner *et al*., 2003). Polymorphism in MHC genes is maintained through mate choice (Eizaguirre *et al*., 2009), where females choose males particularly according their number of MHC alleles in order to create an optimal number of different MHC alleles in their offspring (Milinski, 2003; Reusch *et al*., 2001; Smith & Spence, 2013). The MHC based mate choice and MHC optimum has also proven important for lifetime reproductive success (Kalbe *et al*., 2009) and survival (Wegner *et al*., 2008).

The availability of molecular and immunological tools, the possibility to breed and manage the fish as well as several of their parasites in the lab and the wide knowledge of its ecology make sticklebacks an ideal model system to study the host parasite coevolution.

Schistocephalus spp.

Schistocephalus is a genus within the pseudophyllidean cestodes, where currently at least four species have been described: *S. solidus* (Müller 1776), *S. pungitii* (Dubinina, 1959), *S. nemachili* (Dubinina, 1959), and *S. cotti* (Chubb *et al*., 2006). A few other members of this genus have been described, but their status is highly doubted: *S. fahmi* (Gagarin, Chertkova & Vshchivstev 1966), *S. rhynchichthydis* (Diesing, 1863) and *S. thomasi* (Garoian, 1960).

While *S. solidus* has been used as model system in parasitology and evolutionary ecology (Barber & Scharsack, 2010; Hammerschmidt & Kurtz, 2009), much less is known about the other *Schistocephalus* species.

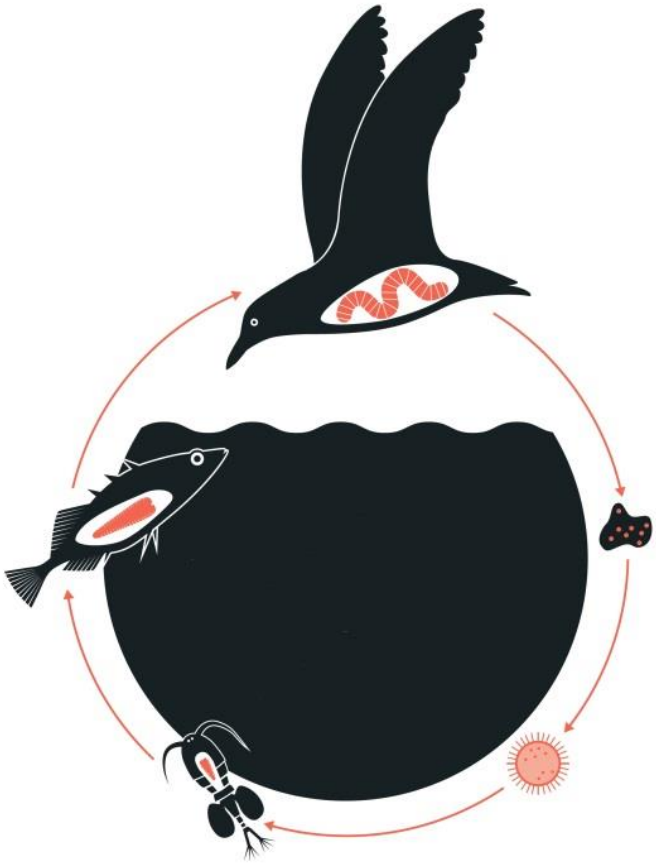

Figure 2: Life cycle of *Schistocephalus solidus* (© Jelka Lerche/ZEIT Grafik)

Schistocephalus solidus reproduces sexually in the gut of piscivorous birds. Eggs are released with the bird's feces into the water, where they hatch into free-swimming larvae. If these larvae are ingested by cyclopoid copepods, they develop into procercoids. Infected copepods have to be eaten by a three-spined stickleback, where the parasite migrates through the intestinal wall of the fish into the body cavity and develops into a plerocercoid. The life cycle is completed, when a bird preys on an infected stickleback. *S. pungitii* has a similar life cycle, but can only infect nine-spined sticklebacks as second intermediate hosts.

S. solidus has a complex life cycle involving three different hosts (Figure 2). It reproduces sexually in the intestine of piscivorous birds – their final hosts. Eggs are then released into the water with the bird's feces, where they develop and hatch into free swimming coracidia (Smyth, 1946). These coracidia infect a copepod – the first intermediate host – by trophical transmission and develop into procercoids. When a three-spined stickleback feeds on infected copepods, the tapeworm is transmitted to its second intermediate host where it develops into a plerocercoid and undergoes an enormous growth. The life cycle is completed, when a piscivorous bird feeds on an infected stickleback (Smyth, 1946).

Schistocephalus pungitii uses the same first intermediate and final hosts as *S. solidus*, but is highly specific to nine-spined sticklebacks as a second intermediate host. Analysis of mitochondrial markers showed that the two *Schistocephalus* species exhibit a deep degree of divergence (Nishimura *et al*., 2011).

Both species are simultaneous hermaphrodites that can either outcross or self-fertilize their eggs. The plerocercoids are segmented, with each segment including both male and female reproductive organs (Clarke, 1954; Schjørring, 2009). Self-fertilization has negative fitness consequences (Christen & Milinski, 2003; Christen *et al.*, 2002), i.e. lower hatching rate and infection success. This is also the reason why *Schistocephalus* has been used to study the evolutionary ecology of mixed mating systems (Benesh *et al*., 2014; Lüscher & Milinski, 2003; Milinski, 2006) and the hermaphrodite's dilemma theory (Lüscher & Wedekind, 2002; Schärer & Wedekind, 1999; Schjørring, 2004; Wedekind *et al*., 1998).

The maintenance of the parasite in the lab is possible for all stages of its life cycle. An *in vitro* system is used to mimic the bird's gut (Smyth, 1946; Wedekind, 1997) and therefore replace the final host. Plerocercoids are removed from the fish and worms are placed into net bags in bottles containing a cell culture medium (Smyth, 1946) at 40°C in the dark for approx. one week. The eggs are collected on the bottom of the container and after several washing steps can be stored in tap water at 4°C for several months. The development of larvae inside the eggs can be induced by raising the temperature. After three weeks of incubation at 20°C in the dark, coracidia can be hatched synchronously, which is triggered by a change in the light regime (Dubinina, 1980). The coracidia can then be used to infect copepods (e.g. *Macrocyclops albidus*), which after approx. two weeks are infective to sticklebacks.

This breeding system allows producing offspring of desired crosses and is quite flexible, regarding the number of plerocercoids: single plerocercoids for self-fertilized offspring (Benesh *et al*., 2014; Christen & Milinski, 2003; Christen *et al*., 2002), multiple mating

partners, sequential mating (Andreou & Benesh, 2014) or even cutting the plerocercoids and mating both halves with different partners is possible (Weinreich *et al.*, 2014). This allows breeding the same genotype of the parasite multiple times with different partners therefore controlling for genotype-specific effects. Furthermore an *in vitro* system to replace the copepod stage has been developed, which facilitates manipulations on this stage of the life cycle (Jakobsen *et al*., 2012).

Stickleback – Schistocephalus as a model system

The three-spined stickleback – *S. solidus* system has become an important model system in the study of host-parasite coevolution (Barber & Scharsack, 2010). Important for this is the circumpolar distribution of both species, the possibility to study the effects of a macroparasite on a vertebrate host and of course the facilitation of *in vitro* breeding of *S. solidus*. Such studies include field-observational and experimental approaches, which combined led to a deep knowledge about the effects of infections with *S. solidus* on many host traits. These include the effects of infection on shoaling and feeding behavior (Barber & Huntingford, 1995; Milinski, 1984; Tierney, 1994), anti-predator behavior (Barber *et al*., 2004; Giles, 1983, 1987a, 1987b; Milinski, 1985; Tierney *et al*., 1993) and host reproduction (Arme & Owen, 1967; Bagamian *et al*., 2004; Heins & Baker, 2008; Heins *et al*., 2010; Heins *et al*., 1999; Heins, 2012; Macnab *et al*., 2011).

The availability of many tools to assess the stickleback immune system also facilitated studies that investigated the effects of *S. solidus* infections on the stickleback immune system and possible immune mechanisms of the parasite to manipulate this (Franke *et al*., 2014; Scharsack *et al*., 2004; Scharsack *et al*., 2013; Scharsack *et a*l., 2007).

In summary, the close adaptation and high specificity of *Schistocephalus* to its stickleback hosts provides a unique opportunity to investigate interactions between parasites and hosts. This thesis addresses adaptations of *Schistocephalus* that aid in host exploitation and the maximization of parasite fitness and how these are possible drivers in parasite speciation. Using a macroparasite and its obligate second intermediate host allows us to investigate host specificity and virulence in a system, where we are able to assess individual parasite fitness as well as the consequences of infection in the fish host.

Thesis outline

In my thesis I investigated the host specificity of the parasites *Schistocephalus* spp. and the adaptation to their second intermediate hosts, sticklebacks. This work is structured into four chapters, which are written in the form of manuscripts and include separate introductions, methods, results and discussions. Chapter II has been published; chapter I, III and IV will be submitted shortly. Chapter IV was prepared in collaboration with Noémie Erin and will also be included in her PhD thesis. My contribution to each chapter can be found in detail in the section of author contributions of this thesis.

Chapter I

This chapter examines the question on where the establishment of *Schistocephalus* spp. fails in incompatible host-parasite combinations. Three different fish species (one of them being the specific host) were exposed to *S. solidus* and the infection was monitored histologically at five different time points from 14 hours to two weeks post-exposure.

In an additional experiment, three-spined sticklebacks were exposed to the incompatible parasite *S. pungitii* at four different time points after exposure to their specific parasite *S. solidus* to test, whether the manipulation of the immune response by the specific parasite allowed a superinfection with the incompatible parasite. Fish were dissected four weeks after exposure and checked for presence of parasites.

Chapter II

Schistocephalus solidus and *S. pungitii* exhibit a similar life cycle sharing the same range of final hosts (piscivorous birds) and first intermediate hosts (cyclopoid copepods). Each species is highly specific on the level of the second intermediate host (either three- or nine-spined sticklebacks respectively). As it is very likely that the two species encounter each other in the final host, where they reproduce sexually, hybridization would be possible. We thus investigated the consequences of hybridization on host specificity and fitness of the hybrids.

The two *Schistocephalus* species were hybridized using an *in vitro* breeding system and fitness relevant traits of the hybrid offspring (outcrossing rate, hatching rate, infection rate in copepods and both stickleback species) and the parental lines were measured throughout their life cycle.

Chapter III

As the results from our previous study (Chapter II) demonstrate, hybrids of *Schistocephalus solidus* and *S. pungitii* show an expanded host range and no obvious fitness disadvantages. Therefore, we investigated possible mechanisms of prezygotic isolation that would lead to the observed deep degree of divergence between these two *Schistocephalus* species and keep them separated.

In a series of experiments we tested if spatial constrains would prevent the parasites from meeting in a natural host, if barriers to hybridization evolved in sympatric populations or if assortative mating was a possible prezygotic barrier in this system. We infected two different bird species simultaneously with the two *Schistocephalu*s species and localized the parasites in the digestive tract, collected feces and typed them using microsatellite markers to estimate hybridization rate. In another experiment we used an *in vitro* system to conduct a mate choice experiment, where a focal worm could choose between a con-specific and worm from a different species as a mating partner.

Chapter IV

In natural communities, hosts are rarely infected with only one parasite. Parasites in multiple infections can encounter intraspecific competition over host resources and host manipulation. This can influence the dynamics of host-parasite interactions with consequences for not only host fitness but also parasite virulence and fitness.

In an experimental approach we tested how intraspecific interactions between two different parasite strains (one high-virulent and one low-virulent strain) alter the parasite performance. Three-spined sticklebacks were infected with one low-virulent and one high-virulent *S. solidus* simultaneously and individual parasite performance and host nutritional and immune status were measured 8 weeks post exposure. As a control we exposed fish to either two parasites with the same level of virulence, one parasite of each strain and used an unexposed control group.

Chapter I

Host specificity in the *Schistocephalus*-stickleback system

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Abstract

The helminth parasites *Schistocephalus solidus* and *S. pungitii* both show an extraordinary degree of host specificity at the level of the second intermediate host. Both species are only able to successfully infect one particular host species, three- or nine-spined sticklebacks respectively.

We exposed three different fish species (three-spined sticklebacks (*Gasterosteus aculeatus*), nine-spined sticklebacks (*Pungitius pungitius*), as well as zebrafish (*Danio rerio*)) to *S. solidus* and monitored the infection process histologically. Our results show that *S. solidus* can establish in both three-spined sticklebacks (their specific host) and in nine-spined sticklebacks (the incompatible, but closely related host). However, in nine-spined sticklebacks they become encapsulated by a layer of fish immune cells after approximately one week and are cleared two weeks after exposure. In zebrafish it seemed that the parasite was not able to reach the body cavity of the fish at all.

In an additional experiment, we tested if the known immune manipulation by *S. solidus* in three-spined stickleback would facilitate the infection with the incompatible parasite *S. pungitii*. We therefore performed simultaneous and sequential experimental infections with both parasite species in three-spined sticklebacks at different time points. These would resemble different stages of immune manipulation by the specific parasite. In no case could we detect a superinfection with the incompatible parasite.

Our results indicate that the degree of host specificity in *Schistocephalus* is extremely high and most likely due to the inability of the parasites to avoid attacks by the immune system of the incompatible hosts.

Introduction

Parasites vary in the number of hosts they can infect successfully. This continuum can range from highly host-specific parasites that are only able to infect one host species, or even only a certain host strain, to broad generalist parasites that can infect a wide range of hosts. In studies of host specificity, often a number of host species are screened for parasites which can create a bias in both directions. A parasite can be described as highly host-specific because it is only found within certain hosts, even though other hosts exist that were just not yet detected. On the contrary, insufficient knowledge of the parasite may lead to several species being described as one, even though they may be entirely independent. The emergence of a variety of molecular tools now allows for differentiation of cryptic parasite species, making studies on parasite host range even more complex (Miura *et al*., 2006; Poulin & Morand, 2004; Poulin & Keeney, 2008).

Host specificity is an important characteristic of a parasite and is affected by several factors. First, ecological factors determine which host species are actually encountered by the parasite. Then, physiological factors (e.g. the parasite's ability to evade the host's immune defence (van Baarlen *et al*., 2007)) determine if the parasite can infect this given host. This has also been described in an "encounter and compatibility filter" by Combes (1991). Parasites might be able to infect this host, but then fail to develop (Combes, 2001; Randhawa *et al*., 2007) or fail to be successfully transmitted to the next host (e.g. in dead-end hosts).

Parasites can experience a trade-off between host specificity and their ability to successfully exploit hosts, leading to a negative correlation between generalism and infection intensity and prevalence (Poulin, 1998; Garamszegi, 2006). Furthermore, highly specialized parasites may have a lower likelihood of jumping to a new host species than more generalist ones (Cleaveland *et al*., 2001). This adaptation to a specific host may change quite rapidly as experimental studies have shown that artificial transfer to a new host species can result in losing the ability to infect original host species (Agrawal, 2000; Ebert, 1998).

Garamszegi (2006) has shown a negative correlation between host range and peak parasitaemia (a proxy for virulence) where virulent parasites are more host-specific than more benign ones. It should be noted that this is not a general pattern that occurs in all systems but nevertheless has been shown multiple times (Gandon, 2004; Regoes *et al*., 2000; Woolhouse, 2001). This argument can also be reversed: more generalist parasites have more opportunities to infect hosts and can become more virulent since they do not pay a cost for virulence (Kirchner & Roy, 2002).

So what is the advantage of being host-specific? It has been suggested that highly specialized parasites can adapt faster to their host and so could replace more generalist species over time (Whitlock, 1996). This more tightly linked coevolution can ultimately lead to speciation in parasites (Duffy *et al*., 2007; Henry *et al*., 2008). However, specialization is not necessarily a one-way road; parasites are capable of evolving in either direction regardless of their ancestral state (Johnson *et al*., 2009).

One example of parasites with a very high degree of host specificity is found in helminths. *Schistocephalus solidus* and *S. pungitii* are cestodes with a complex life cycle and show an extraordinary degree of host specificity at the level of the second intermediate host – the three-spined stickleback (*Gasterosteus aculeatus*) or the nine-spined stickleback (*Pungitius pungitius*). The two species are closely related and a recent study suggest that they separated approximately 20-25mybp (Nishimura *et al*., 2011).

Most helminths including *Schistocephalus* spp. infect their hosts via the oral route (Mulcahy *et al*., 2004). *Schistocephalus solidus* and *S. pungitii* need between 2h and 2d to reach the body cavity of the stickleback host (Clarke, 1954; Dubinina, 1980). The migration within the intestinal lumen and through the intestinal wall happens relatively rapidly. In general, it seems that the parasites have to be fast, especially in the stomach; otherwise they will be harmed and degraded by the acidic stomach environment (Hammerschmidt & Kurtz, 2007). The successful crossing of the mucosal barrier seems to be a crucial step in the infection process in various parasites of medical or veterinary importance (Mulcahy *et al.*, 2004).

During the infection process in the stickleback host, *Schistocephalus* loses its cercomer (a caudal appendage of the larval cestode) and outer membrane before the penetration of the intestinal wall. Therefore the underlying tegument, containing mostly WGA-binding sugars (GlcNAc, sialic acids) with its microtriches is already exposed at this stage (Hammerschmidt & Kurtz, 2005b, 2007; Schmidt & Peters, 1987). These WGA-binding sugars have been suggested to help the parasite in evasion of the stickleback immune system (Hammerschmidt & Kurtz, 2005b; Schmidt & Peters, 1987). Other functions of these microtriches include uptake of nutrients, parasite movement and, in hookless tapeworms, anchoring to host tissue (Mehlhorn & Armstrong, 2001). Microtriches are also possibly important for the penetration of the intestinal wall as it was shown in related species such as *Taenia* sp. and *Echinococcus* sp. (Barker, 1970; Heath, 1971).

During the development from procercoid to plerocercoid in *Schistocephalus*, a change in surface carbohydrates occurs, induced by bile fluid of the fish host (Marwaha *et al*., 2013),

which could protect against enzymatic digestion in the intestines as well as immune defence in the body cavity of sticklebacks (Hammerschmidt & Kurtz, 2005b).

Even though sticklebacks seem to be unable to avoid infected prey behaviourally (Wedekind & Milinski, 1996), the fish immune system appears to be able to clear the infection within the first two weeks (Scharsack *et al*., 2007), or reduce parasite growth, which also incurs costs for the stickleback (Hammerschmidt & Kurtz, 2005a; Kurtz *et al*., 2004, 2006).

Most parasites use some form of immune evasion to persist in their hosts. In trematodes (i.e. schistosomes) attachment of host cell surface antigens is believed to defend parasite against immune attack (molecular mimicry), a phenomenon less thoroughly studied in cestodes (Smyth & McManus, 1989). At least in *Ligula intestinalis* it was observed that the parasite attaches protective host proteins onto its the surface (Hoole & Arme, 1983; Williams & Hoole, 1995), indicating a similar mechanism for molecular mimicry in cestodes. Some cestodes (e.g. *Taenia pisiformis*) can also release substances which inhibit proteolytic enzymes such as trypsin and chymotrypsin (Németh & Juhász, 1980) or induce immunosuppression (Rickard, 1986). Also, modifications by parasites to cell differentiation, macrophage activation, responsiveness to mitogens, cytotoxicity and complement activation have been reported (Rickard, 1986). *Schistocephalus solidus* itself has been shown to modulate the stickleback's immune response, probably through excretory products (Scharsack *et al*., 2004; Scharsack *et al*., 2013).

In a study by Orr and colleagues (1969) it was shown that *S. solidus*, which is specific to three-spined sticklebacks, can establish in the body cavity of nine-spined sticklebacks but fails to reach an infective size. In nine-spined sticklebacks the pleroceroids grew slower than in three-spined sticklebacks and were cleared by the host after 10 days. Another study (Bråten, 1966) showed plerocercoids transplanted from three- to nine-spined sticklebacks stopped developing and later on showed destruction of the tegument.

Taken together, these results show a high degree of host specificity in *S. solidus* and furthermore a high immunological specificity for the second intermediate host. The failure to establish an infective stage of the parasite in the fish host is likely due to the interaction between the tapeworm and the fish immune system. This finding is supported by a study that indicated that *S. solidus* actively manipulates the immune response in the three-spined stickleback (Scharsack *et al*., 2004).

If a stickleback is first infected with its specific tapeworm species, a superinfection with an incompatible tapeworm may establish easier, since the host immune system is already altered by the first parasite. In sequential infections with two *S. solidus*, it has been shown that the

second parasite had a better chance of survival and better growth than the first parasite (Jäger & Schjørring, 2006), indicating some kind of facilitation by immune manipulation of the first parasite.

For this purpose we infected three-spined sticklebacks with their specific tapeworm *S. solidus* and exposed them at different time points to *S. pungitii*. The time points should reflect the following stages: i) initial infection (d0), ii) establishment of the parasite in the body cavity (d7 and d14) and iii) the first parasite reached an infective stage and is ready to be transmitted to the final host (d60).

Additionally, we infected three- and nine-spined sticklebacks as well as the nonrelated zebrafish (*Danio rerio*) with *S. solidus* to monitor the infection process in specific and non-specific host species at five different time points histologically.

Methods

Model system

Schistocephalus solidus and *S. pungitii* are two closely related cestodes with a similar complex life cycle involving three different hosts. Both species reproduce sexually in their final hosts, piscivorous birds. Afterwards, eggs are released with the bird's feces into the water, where free-swimming larvae can hatch and trophically infect the first intermediate host, cyclopoid copepods. There, the parasites develop into procercoids. If an infected copepod is eaten by a stickleback (*G. aculeatus* for *S. solidus* and *P. pungitius* for *S. pungitii*), they can migrate into the fish's body cavity and develop into plerocercoids. When birds feed on parasitized sticklebacks, the parasites reach their final host and the life cycle is completed.

It is possible to complete the parasites' life cycle in the lab, replacing the final host with an *in vitro* breeding system (Smyth, 1946). This allows for breeding distinct combinations of parasites that can be used for individual and controlled experimental exposure of copepods and sticklebacks.

Histological analysis of establishment in the fish host

For the histological analysis of the establishment of *S. solidus* in its specific and two non-specific fish hosts, we used lab-bred offspring of three different fish species: *G. aculeatus* (originating from the Große Plöner See, Germany, 54° 09' 21" N, 10° 25' 50" E), *P. pungitius* (originating from the Neustädter Binnenwasser, Germany, 54° 06' 41" N, 10° 48' 33" E) and *D. rerio* (which we purchased in a pet shop).

The fish were starved for one week, isolated into individual 2l tanks and then fed with *S. solidus* infected copepods in order to achieve a total number of ~50 procercoids for exposure per fish. At each time point (14h, 18h, 24h, 1 week, 2 weeks) we killed one fish of each species with an overdose of MS222 and immediately fixed them in 4% formalin. Subsequent processing of the fish for histological screening was carried out according to Hammerschmidt and Kurtz (2007).

Simultaneous or sequential infection experiment

Lab-cultured copepods (*Macrocyclops albidus*) were individually exposed to *S. solidus* or *S. pungitii*. The *S. solidus* parasites were laboratory-bred offspring from one sibship that originated from a population in Skogseidvatnet (Norway, 60° 14' 38" N, 05° 54' 51" E). *S. pungitii* parasites were laboratory bred offspring from one sibship that originated from a population in Obbola (Sweden, 63° 39' 22" N, 20° 17' 27" E). The three-spined sticklebacks were laboratory-bred offspring that originated from two sibships from the Große Plöner See (Germany, 54° 09' 21" N, 10° 25' 50" E).

Three-spined sticklebacks were initially exposed to their specific parasite *S. solidus* and then at different time points to *S. pungitii*. Each of the four different treatment groups consisted of 30 individual fish. Exposure of fish was carried out by feeding one singly infected copepod to each fish. In the first group (d0), fish were simultaneously exposed to *S. solidus* and *S. pungitii*. In the other treatments (d7, d14 and d60) fish were kept in groups of 15 individuals between the exposures.

Four weeks after exposure to *S. pungitii*, the fish were killed with an overdose of MS222 (tricaine methanesulfonate, 1mg/ml) followed by a subsequent cervical incision, and dissected to check for the presence of parasites.

From each parasite, including the parental parasite generation, a tissue sample was taken and DNA extracted using the DNeasy Kit from Qiagen. Using microsatellites (Binz et al., 2000) we confirmed the parentage and species for each parasite.

Results

Histological analysis of establishment in the fish host

We screened fish from the three different species which have been exposed to *S. solidus* histologically for parasite presence in stomach and intestinal lumen, intestinal wall and body cavity (Table I-1). No statistical analysis was possible, since the exact number of procercoids (~50 exposed per fish) was unknown and only one fish per species and time point was used. The main goal of this experiment was a qualitative estimate: to find out where and when the establishment of *S. solidus* in the "wrong" host species fails.

Our results showed that in its specific host (*G. aculeatus*), the parasite reached the body cavity already within 14h post exposure (pe). The only time point where parasites could be detected within the intestinal lumen and intestinal wall of three-spined sticklebacks was after 24h pe. During all five time points parasites could be found in the body cavity of *G. aculeatus*.

In the closely related *P. pungitius* no parasites could be detected in the stomach lumen, but were detected in the intestinal lumen and intestinal wall between 14h and 24h pe. In this fish, *S. solidus* also managed to reach the body cavity within 14h pe. After 1 week pe *S. solidus* in the non-host *P. pungitius* were surrounded by several layers of host cells, most likely granulocytes, and the parasite's surface showed clear signs of disintegration (Figure I-1). After 2 weeks pe no intact *S. solidus* larvae were detected anymore in *P. pungitius*, but some structures in the body cavity remained which may be residual encapsulated and degraded parasites. Disintegration of the parasite's surface was only seen in parasites that were already in the body cavity of *P. pungitius* and did not appear before 1 week pe (Table I-1).

In *D. rerio*, not a single parasite could be detected in any of the screened organs.

Table I-1: Summary of the number of *S. solidus* found in the three different fish hosts at five different time points

Each individual fish was exposed to ~50 procercoids. We divided the total number of parasites found in each fish into parasites found in the gastrointestinal tract (stomach or gut) or within the body cavity. We also counted how many parasites within the body cavity of the fish appeared encapsulated.

In *P. pungitius* 2 weeks pe 8 structures within the body cavity were found that could possibly be remainders of encapsulated and disintegrated parasites (marked with *).

	n gastrointestinal tract	***n body cavity***	***n encapsulated***
G. aculeatus			
14h	7	5	0
18h	0	8	0
24h	1	7	0
1 week	0	12	0
2 weeks	0	15	0
P. pungitius			
14h	0	14	0
18h	0	8	0
24h	0	20	0
1 week	0	17	11
2 weeks	0	8*	8*
D. rerio			
14h	0	0	0
18h	0	0	0
24h	0	0	0
1 week	0	0	0
2 weeks	0	0	0

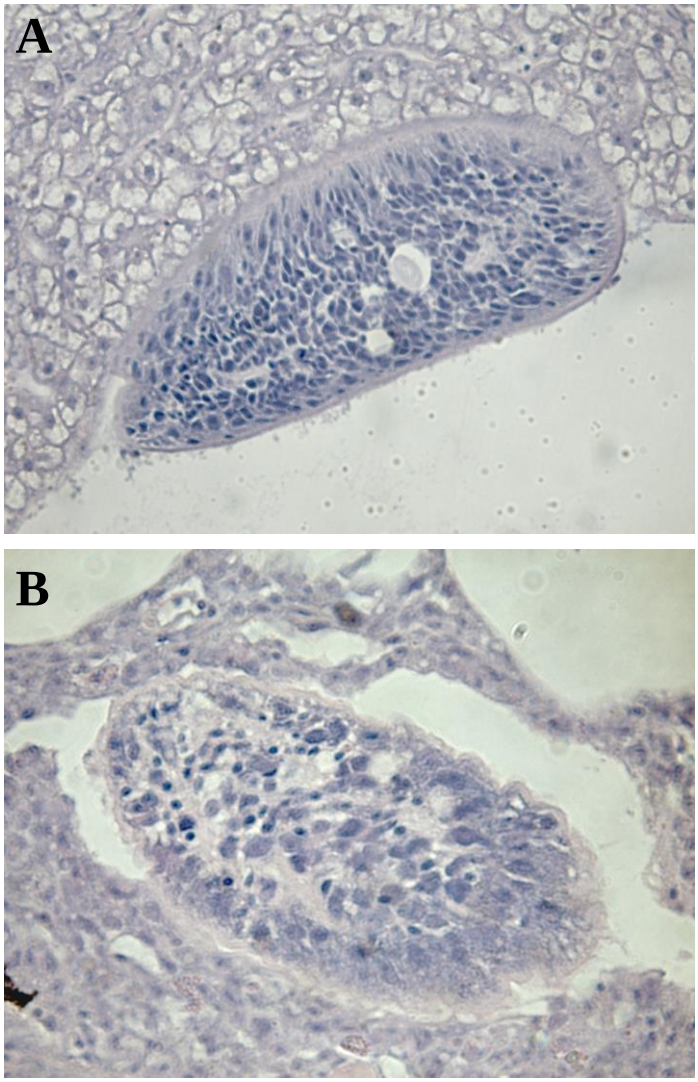

Figure I-1: *Schistocephalus solidus* plerocercoids 7d post exposure in the body cavity of *G. aculeatus* (A) and *P. pungitius* (B)

In its specific host (A), the parasite lies next to the liver in the body cavity. Its tegument appears smooth and fully intact. In the non-specific fish host (B), *S. solidus* seems to be surrounded by a layer of fish immune cells and its tegument appears disintegrated and in the process of being dissolved.

Simultaneous or sequential infection

Dissection of the fish infected with both parasites at the four different time points revealed only one fish that harbored two parasites in the d60 treatment. All other fish were only singly infected or uninfected (Table I-2). From each plerocercoid recovered, a tissue sample was taken for DNA extraction and analysis to confirm the species. In all cases – including the double infected fish in the d60 treatment – the plerocercoids were *S. solidus*. In no case could

we find a *S. pungitii*. The double infection is most likely the result of an overlooked double infection in the copepod.

Table I-2: Summary of the number of fish exposed and infected in simultaneous or sequential exposure of *Gasterosteus aculeatus* to *Schistocephalus solidus* and *S. pungitii*

Three-spined sticklebacks were exposed to the specific parasite S. solidus and at different time points to the incompatible parasite S. pungitii. A simultaneous exposure to each parasite was carried out on d0. In the other treatments the fish were first exposed to one *S. solidus*, and after 7 (d7), 14 (d14) or 60 (d60) days additionally to one *S. pungitii*.

time point	*n exposed*	*n dissected*	*n infected with 1 plerocercoid*	*n infected with 2 plerocercoids*
d0	30	29	5	0
d7	30	30	9	0
d14	30	30	8	0
d60	30	30	11	1

Discussion

How is this high host specificity in *Schistocephalus* maintained? Where and when does the parasite fail to establish in non-specific combinations? Following the establishment of the parasite in different fish hosts histologically, we could show that *S. solidus* manages to reach the body cavity of *G. aculeatus* and the closely related *P. pungitius*, but not *D. rerio*. This was in accordance with earlier studies (Orr *et al*., 1969) which have also shown that the parasite can reach the body cavity of *P. pungitius*, but does not manage to reach an infective size and is cleared by the host within a few days after infection.

Why can't *S. solidus* reach the body cavity of *D. rerio*? It seems that in *D. rerio*, *S. solidus* can't reach the intestine or body cavity of the fish. Possibly the parasite is already digested in the stomach of the fish, if the protection from the acidic stomach environment fails in this non-specific host. It is possible that the parasite's surface carbohydrate composition plays a crucial role during the establishment phase. The composition of the surface of *S. solidus* has been shown to variable between sibships and to be correlated with infectivity and growth (Hammerschmidt & Kurtz, 2005b). This can indicate, that the surface carbohydrates still

allow *S. solidus* to migrate through the intestinal wall and reach the body cavity of the incompatible even though closely related host, but not the non-related *D. rerio*. Orr and colleagues (1969) also exposed an unrelated fish species (*Barbus* sp.) to *S. solidus* and found similar results, as the parasite also did not enter the body cavity in this fish species. They also concluded that the procercoids were not able or stimulated to penetrate the intestinal wall.

However, once it has reached the body cavity, nine-spined sticklebacks seem to target *S. solidus* which appears encapsulated and disintegrated in our histological analyses. Only the close coevolution between two specific species seems to allow the parasite to evade the stickleback immune response. If the acquisition of host molecules on the parasite surface is the crucial step in masking itself from the host immune system, one could imagine that the incompatible parasite fails at this step, being left unguarded from the immune response. Since hybrids between the two species are infective to both stickleback hosts (Henrich *et al*., 2013), it is possible that this trait is inherited additively, allowing molecular mimicry for both fish species. One cannot exclude that other parts of the parasites' surface structure, such as carbohydrates (Hammerschmidt & Kurtz, 2005b, Schmidt & Peters 1987) are also intermediate in hybrids, allowing the parasites to develop successfully in both hosts.

The second experiment with simultaneous or sequential infections with both parasite species in three-spined sticklebacks showed no obvious facilitation of an infection with an incompatible parasite.

If one specific parasite manipulates the stickleback's immune response in order to escape clearance in the body cavity, this does not appear to affect the successful establishment of a non-specific parasite species, neither during the infection and establishment phase, nor when the first and specific parasite has reached an infective size. Therefore, it seems likely that the incompatible parasite is unable to escape the immune response of the fish host, potentially as a result of its inability for molecular mimicry in the incompatible host.

A study on the *in vitro* response of head kidney leucocytes from three-spined stickleback to *S. solidus* and *S. pungitii* antigens have shown no strong differences between the two parasite species (Franke *et al*., 2014). The only difference was shown in cell viability, which was elevated in cultures with *S. pungitii* antigens, while remaining at control levels in cultures with *S. solidus* antigens (Franke *et al*., 2014). This might also indicate a closer adaptation of the immune system of three-spined stickleback to the specific parasite species.

What are the consequences of this high specificity? It seems that *Schistocephalus* is a parasite that requires a very tight interaction with its second intermediate host. Even though both parasites and fish hosts do occur in sympatry, infections with the incompatible parasite

species have not yet been detected. This may be partially due to an observation bias because the parasite species is rarely confirmed with molecular tools. Rather, the species of fish it is found in serves as the decisive criterion for species distinction, as both parasite species are very similar morphologically (Dubinina, 1959). Nevertheless, being able to escape the immune system of the fish host only seems to work in the specific host-parasite combination. This close coevolution could be necessary for being able to exploit the host in such a manner as *Schistocephalus* does, paying the cost of not being infective to any other fish species.

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Chapter II

Hybridization between two cestode species and its consequences for intermediate host range

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Abstract

Background: Many parasites show an extraordinary degree of host specificity, even though a narrow range of host species reduces the likelihood of successful transmission. In this study, we evaluate the genetic basis of host specificity and transmission success of experimental F1 hybrids from two closely related tapeworm species (*Schistocephalus solidus* and *S. pungitii*), both highly specific to their respective vertebrate second intermediate hosts (three- and nine-spined sticklebacks, respectively).

Methods: We used an in vitro breeding system to hybridize *Schistocephalus solidus* and *S. pungitii*; hybridization rate was quantified using microsatellite markers. We measured several fitness relevant traits in pure lines of the parental parasite species as well as in their hybrids: hatching rates, infection rates in the copepod first host, and infection rates and growth in the two species of stickleback second hosts.

Results: We show that the parasites can hybridize in the in vitro system, although the proportion of self-fertilized offspring was higher in the heterospecific breeding pairs than in the control pure parental species. Hybrids have a lower hatching rate, but do not show any disadvantages in infection of copepods. In fish, hybrids were able to infect both stickleback species with equal frequency, whereas the pure lines were only able to infect their normal host species.

Conclusions: Although not yet documented in nature, our study shows that hybridization in *Schistocephalus* spp. is in principle possible and that, in respect to their expanded host range, the hybrids are fitter. Further studies are needed to find the reason for the maintenance of the species boundaries in wild populations.

Background

In interaction with their host organisms, many parasite taxa show an extraordinary degree of specificity, which is often regarded as indication of a long co-evolutionary history. In fact, parasites with a rather narrow range of suitable host species have been shown to be better adapted to sympatric host populations than generalist parasites (Lajeunesse & Forbes, 2002; Poulin, 1998). However, the actual advantage of being restricted to only one or very few host species is still elusive. Particularly for parasites with complex life cycles, a narrow host range can be very disadvantageous since it decreases the probability for transmission when suitable host species are rare. Therefore, a good strategy for a parasite would be to become optimally adapted to one host species, but capable of a host-switch to avoid extinction when under changing ecological conditions the specific host disappears.

One possibility for a rather fast expansion of the host range could be the introgression of host compatibility genes by hybridization between closely related parasites species (Detwiler & Criscione, 2010). Furthermore, this might also be a way to escape extinction, since specialization has been suggested as a one-way street (Leroux, 1954; Nosil, 2002). Such a scenario is particularly conceivable in macroparasites with complex life cycles, where two parental species are highly specific to different intermediate hosts, but share a common final host where sexual reproduction takes place.

In all major taxa of helminth parasites, hybridization has been found in nature or been demonstrated between sympatric species in laboratory experiments. Most examples have been described in digeneans (Agatsuma *et al*., 2000; Huyse *et al*., 2009; Leroux, 1954; Morgan *et al*., 2003; Southgate *et al*., 1976; Steinauer *et al*., 2008; Taylor, 1970; Tchuem Tchuente *et al*., 1997; Wright *et al*., 1974), but there is also evidence from cestodes (Okamoto *et al*., 2010), monogeneans (Kuusela *et al*., 2007; Schelkle *et al*. 2012) and nematodes (Grabner *et al*., 2012; Martín-Sánchez *et al*., 2005). Testing whether or not hybridization may increase fitness by extending the range of suitable (intermediate) host species requires experimental studies to determine transmission success in the different stages of a parasite life cycle. Particularly in schistosomes, several studies have shown that hybrids between two species or strains inherited the ability to develop in both specific host snails of the respective parental lines and retain this increased host range over several generations (Huyse *et al*., 2009; Mutani *et al*., 1985; Pagès *et al*., 2002). Also for behavioral traits related to transmission, like diurnal cercarial shedding patterns (Théron & Combes, 1988) and specificity in host-finding behavior (Kalbe *et al*., 2004), hybrids of different *Schistosoma*

mansoni strains have been shown to have trait values intermediate between the parental strains.

Schistocephalus solidus, a cestode with a complex life cycle, is extremely specific for its second intermediate host, infecting only the three-spined stickleback *Gasterosteus aculeatus*. This system has become a model system for experimental studies on the evolutionary ecology of host-parasite interactions (reviewed by e.g. Barber & Scharsack, 2010; Hammerschmidt & Kurtz, 2005). *Schistocephalus pungitii* is closely related to *S. solidus*, but uses the nine-spined stickleback *Pungitius pungitius* as second intermediate host, and shows the same host specificity at this level (Dubinina, 1980). Both parasites potentially share the same final hosts (Dubinina, 1980) and often occur in sympatry (Morozińska-Gogol, 2006; Zander *et al*., 1999). Hence, natural encounters between adults of the sister species are plausible, making hybridization a possibility. However, a recent study by Nishimura and colleagues (2011) shows a deep lineage divergence in the *Schistocephalus* genus, suggesting that separation of both species occurred shortly after the speciation of their respective stickleback lineages circa 20–25 million years ago. Hybrids have not been observed in nature yet and earlier experiments have shown that both *Schistocephalus* species are not able to infect the reciprocal intermediate hosts. Additionally, plerocercoids transplanted between three- and nine-spined sticklebacks stopped developing and later on showed destruction of the tegument (Bråten, 1966; Orr *et al*., 1969). Thus, these two species exhibit a high immunological specificity for their second intermediate host.

Many parasites undergo extensive growth in their final host, relative to that in their intermediate hosts (Benesh *et al*., 2013). However, *Schistocephalus* undergoes enormous growth in its second intermediate host. The worm is extensively challenged by the host's immune system (Scharsack *et al*., 2004; Scharsack *et al*., 2007), so it is possible that this rapid growth is facilitated by highly specific adaptations to the host's immune system. At least ***in vitro***, the size of the worm is proportional to egg output (Schärer *et al*., 2001; Wedekind *et al*., 1998), suggesting that specificity, growth, and fitness may be tightly linked in this system.

This system offers a unique possibility to investigate host specificity in two closely related parasite species with complex life cycles. It is likely that both parasite species meet in a bird's gut for reproduction, which could facilitate interspecies mating. Both parasites are simultaneous hermaphrodites and capable of self-fertilization (selfing). Since selfing is costly for the parasite in all stages of its life cycle (Christen & Milinski, 2003; Christen *et al*., 2002;

Milinski, 2006; Schjørring, 2004), hybridization would seem to be a good way to avoid the negative effects of inbreeding when outcrossing is not possible.

The aim of this study was to investigate the possibility of hybridization between the two cestode species of sticklebacks and the consequences of hybridization for host specificity and fitness at all stages of the parasite's life cycle.

Methods

Study system

Schistocephalus solidus reproduces sexually in the intestines of piscivorous birds – their final host. Eggs are then released into the water with the bird's feces, where they hatch into free swimming coracidia (Smyth & McManus, 1989). Copepods ingest coracidia and the worm develops into a procercoid in the copepod body cavity. When a three-spined stickleback feeds on infected copepods, the tapeworm is transmitted to its second intermediate host where it develops into a plerocercoid and undergoes enormous growth. The life cycle is completed when a piscivorous bird feeds on an infected stickleback (Smyth & McManus, 1989). *S. pungitii* shares the main characteristics of this life cycle, but uses *P. pungitius* as a second intermediate host.

The two species of parasite can be maintained in the lab for all stages of their life cycle. Plerocercoids are removed from the fish and can be bred in an *in vitro* system that mimics the bird's gut (Smyth, 1946; Wedekind, 1997). Worms are usually size-matched for breeding, as this limits selfing (Lüscher & Milinski, 2003). After three weeks of incubation at 20°C in the dark, the coracidia start to hatch from eggs (Dubinina, 1980). The coracidia can then be used to infect copepods (e.g. *Macrocyclops albidus*). After approximately two weeks of development in copepods, worms are infective to sticklebacks (Benesh & Hafer, 2012; Clarke, 1954; Hammerschmidt *et al.*, 2009). Figure II-1 shows the life cycle of *Schistocephalus*, the most relevant traits measured in this experiment, as well as the breeding design for hybridizing the two parasite species.

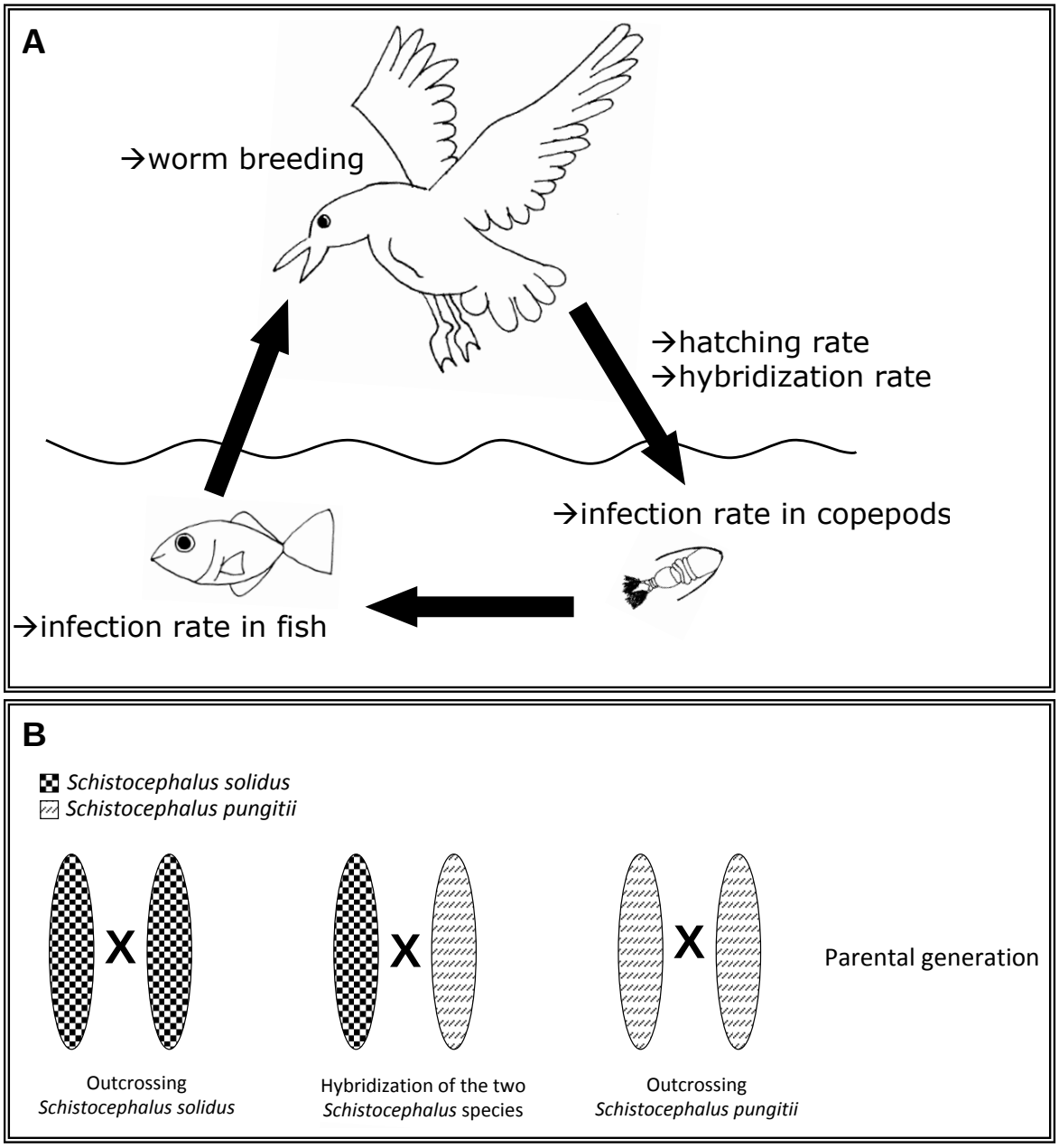


Figure II- 1: Experimental design and measured parameters. A: Life cycle of *S. solidus* and *S. pungitii* and parameters measured in this study. **B**: Experimental breeding design for hybrid worms.

Breeding design and worm origin

Lab-infected sticklebacks originated from two allopatric populations: Skogseidvatnet, Norway (60°31′N, 05°13′E) for three-spined sticklebacks with *S. solidus* and Lebrader Teiche, Germany (54°22′N, 10°42′E) for nine-spined sticklebacks with *S. pungitii*. Fish were dissected and worms were paired for breeding. We used two different sibling families for each worm species (sibships, which refers to offspring from one pair of worms that were obtained from our lab cycle). For each sibship, at least one of the worms was paired with a

conspecific from a different sibship, while at least one was paired with a worm from the other species. This was done so that the genetic composition of hybrid pairs and pure species pairs was similar, so that any observed differences between hybrid and pure groups are likely due to hybridization *per se* rather than random genetic differences between groups. Unfortunately, we were limited in the number of *S. solidus* plerocercoids, so we bred seven pairs in total: one outcrossed *S. solidus* pair, two outcrossed *S. pungitii*, and four potential hybrid pairs.

Breeding conditions

The standardized laboratory breeding system (Smyth, 1946; Wedekind, 1997) was slightly modified in that we diluted the medium with sterile filtered water, which we found was more suitable for *S. pungitii*. *In vitro* cultured, adult worms were transferred into netbags with their respective partner and these netbags put into a bottle containing pre-warmed medium (60% Eagle's Minimal Essential Medium [Sigma] and 40% sterile filtered tap water). The bottles were incubated in a 40°C shaking water bath in the dark for two days. After two days we assumed that reciprocal fertilization had happened (see e.g. Schärer *et al*., 2001) and we isolated single worms in 50 ml tubes containing fresh pre-warmed medium. Eggs were collected from each worm for another three days in the breeding system. All collected eggs were washed with cold tap water (4°C) to prevent any larval development.

Estimation of hatching rate & hybridization rate

The eggs were incubated at 20°C for 21 days in the dark. On day 21, the eggs were exposed to 4 h of light, followed by an 8 h period of darkness and another light period afterwards to stimulate hatching of coracidia. From each worm we aimed to collect 96 coracidia for determining hybridization rates via microsatellite analysis, while the remaining larvae were used to infect copepods. Low hatching rates limited the number of coracidia available in some groups (see Results). From the collected coracidia, DNA was extracted with chelex (after Lüscher & Milinski, 2003) and each individual was typed with microsatellites using six different loci (primers and PCR conditions in (Andris *et al*., 2012; Binz *et al*., 2000) to estimate outcrossing/hybridization rates (see below).

The remaining eggs were left in a 16 hours light/8 hour dark room for another three weeks to ensure that every viable larva hatched. Afterwards, 100 eggs per worm were inspected visually to estimate the number of hatched coracidia.

Exposure of copepods

Cultured copepods (*Marcrocyclops albidus*) (see van der Veen & Kurtz, 2002 for details on cultures) were each exposed to a single coracidium. We aimed to expose 96 copepods per single worm, which could not be achieved in every case because of the low number of hatched coracidia in some worm sibships. The copepods were starved 1 day before exposure and afterwards fed every second day alternatingly with two *Artemia salina* nauplii or ~100 *Paramecium caudatum*. Copepods were checked visually for the presence of procercoids on day 8 and 9 post exposure. Infected copepods were then fed to fish on day 16. By this time, at least in *S. solidus*, worms are essentially fully developed and infective to fish (Benesh, 2010), so infection success in fish is unlikely to be attributable to developmental variation (Benesh & Hafer, 2012).

Infection of sticklebacks

Two German populations of naive lab bred sticklebacks (*G. aculeatus* from Großer Plöner See (54°07′N, 10°24′E) and *P. pungitius* from Lebrader Teiche (54°22′N, 10°42′E)) were used to test the infection success of hybrids and pure parental parasite lines in the second intermediate host. We used an allopatric combination for *S. solidus*/*G. aculeatus*, since we did not have access to enough fish from the sympatric population. Fish were put singly in plastic tanks containing approx. 1 L of water and starved for one day before exposure. Each fish was exposed to a single infected copepod. One day after the exposure the fish were moved in groups to 16 L tanks. The remaining water in the single tanks was filtered to ensure that all copepods were eaten by the fish. Fish were kept at 18°C and 16/8 h light/dark period and were fed three times per week *ad libitum* with frozen daphnids and chironomid larvae.

Nine weeks after exposure the fish were killed with an overdose of MS222, measured, weighed, and the body cavity was opened to remove and weigh worms if present. A tissue sample was collected from each worm for microsatellite typing to check whether it was an outcrossed, selfed or hybrid individual.

Data analyses

We analysed the fitness relevant traits of the parasite separately. Two of the measured traits, hatching rates and outcrossing/hybridization rates, are characteristics of sibships and we analyzed them at this level. A generalized linear model (GLM) with quasi-binomial errors and a logit link function was used to compare hatching rates in the three groups (pure *S.*

solidus, pure *S. pungitii* and hybrids). A similar GLM was used to compare the hybridization rate of hybrid pairs to the outcrossing rate of *S. solidus*. Only *S. solidus* and the hybrids could be compared, because, unfortunately, the microsatellite markers developed for *S. solidus* were not suitable to estimate outcrossing rate in *S. pungitii*, since all our individuals were homozygous across all loci.

Infection rates in copepods and fish were analyzed at the level of individual hosts. To evaluate whether infection rates differ between hybrids and the pure species groups, we fitted GLMs with binomial errors and a logit link function (Wilson & Grenfell, 1997). For infection rates in fish, in addition to the parasite group, we also included fish species (*G. aculeatus* and *P. pungitius***)** as a factor. In some combinations of parasite group and fish species, no fish became infected (see Results). This kind of data structure (i.e. complete separation) causes inflated standard error and confidence interval estimates. Thus, we used the logistf R function (R package "logistf" (Ploner *et al*., 2006) to fit the GLM with penalized likelihood (Heinze & Puhr, 2010).

Finally, an analysis of covariance (ANCOVA) was used to test whether worm weight differs between fish species-worm species combinations while controlling for fish weight at dissection.

All statistical analyses were carried out using R 2.12.2 (R Development Core Team, Vienna). P-values lower than 0.05 were considered significant.

Ethical statement

All animal experiments described were approved by the 'Ministry of Energy, Agriculture, the Environment and Rural Areas' of the state of Schleswig-Holstein, Germany (reference number: V 313–72241.123-34).

Results & Discussion

Hybridization rate / outcrossing rate

Analysis of six different microsatellite loci revealed a hybridization rate of 24 to 49% in three hybrid pairs (a total of 141 typed coracidia). The remaining 76 to 51% were selfed individuals. The outcrossing rate in the *S. solidus* pair was 95% (91 coracidia typed). Both a Fisher's exact test ($P < 0.0001$) and a GLM at the level of sibships ($n=4$, $F_{1,2}= 24.04$, $P =$

0.039) indicated that there were significantly more selfed individuals in hybrid pairs than in conspecific *S. solidus* pairs.

Our results show clearly that hybridization between *S. solidus* and *S. pungitii* from two allopatric populations is possible under laboratory conditions. The estimation of the selfing rate is biased by the fact that only hatched coracidia can be genotyped with microsatellites. The real rate of hybridization or outcrossing remains unclear since the genetic markers cannot be used on unhatched eggs (Lüscher & Milinski, 2003).

Hatching rate

A Fisher's exact test indicated that overall hatching success differed significantly between groups (P = 0.0005). The hybrid pairs had the lowest hatching rate (mean 7.375%, n=4 worm pairs), followed by the *S. pungitii* (15%, n=2) and *S. solidus* (24.5%, n=1). However, the GLM with sibships as units did not indicate significant differences between the three groups (F-test comparing null model with model including treatment effect: $F_{2,4} = 1.72$, P = 0.29). Thus, we conclude that hybrids tend to have lower hatching rates than pure species sibships, but a larger number of sibships must be observed to confirm this difference.

The lower hatching rate in hybrids may be a consequence of the strong inbreeding depression of selfed individuals, as it was also previously shown by Christen *et al***.** (2002) and Schjørring (2004) that the hatching rate of selfed worms was about 4 to 8 times lower than in outcrossed individuals.

Infection rate in copepods

A GLM indicated significant differences between groups (likelihood ratio test with an intercept-only model, $\chi^2_2 = 109.43$, P < 0.001). Worms bred in hybrid pairings showed an infection rate that was between the infection rates of *S. solidus* and *S. pungitii* (Figure II-2). Below we address the possibility that the relatively high proportion of selfed offspring in the hybrid pairs biases the infection rate estimate for hybrids.

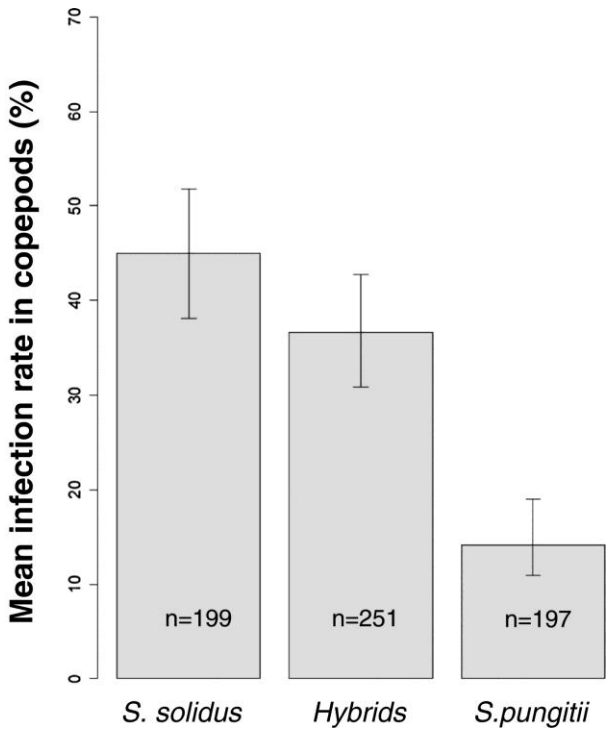


Figure II-2: Mean infection rate in copepods.

Error bars show 95% CI.

Infection rate in fish

When we compare the infection rates in fish (Figure II-3), we see that worm species or fish host alone doesn't have an effect on the infection rate. This was supported by a likelihood ratio test that showed the GLM with an interaction term (fish species x worm group) was significantly better than the simpler model with just the two main effects (χ^2_2 = 25.41, P < 0.001). Microsatellite analysis after removal of the worms from fish showed that most worms from hybrid pairs were hybrids and not selfed individuals. In total, we found five selfed worms among 32 individuals. Again, the infection rate estimates for hybrids might be biased by the unknown proportion of copepods harboring selfed worms that were fed to the fish. We address this issue in the section below.

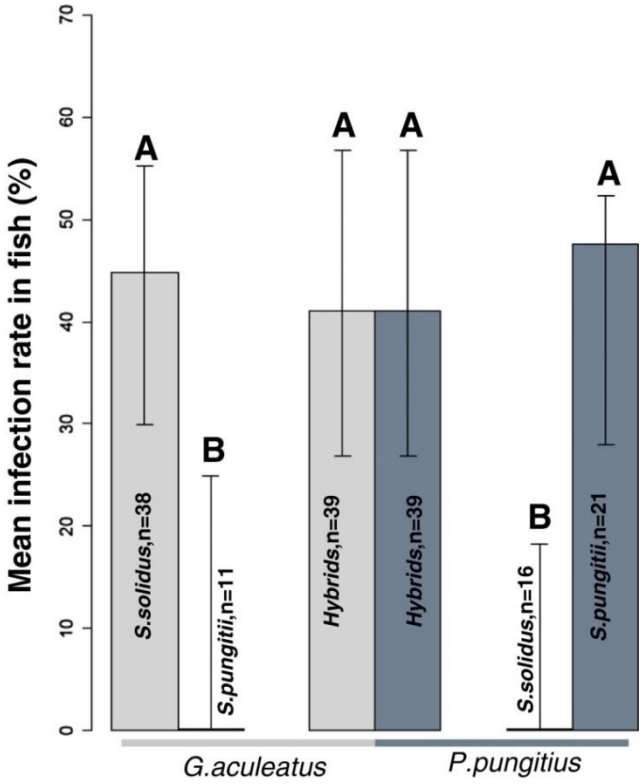


Figure II-3: Mean infection rate in fish.

Error bars show 95% CI. Groups with different letters (**A**, **B**) differ significantly from each other.

In our study, each species of worm was only able to infect its specific host while their non-host stickleback was never infected. Strikingly, the hybrids were able to infect both fish hosts with equal probability, while the pure lines only infected their specific fish host.

Since the hybrids are able to infect both fish hosts at similar rates, they have expanded their host range. If the genes responsible for this trait were purely additive, we would have seen ~20% infection rate of hybrids in fish (i.e. intermediate between the pure lines). Instead, we see a kind of co-dominance where hybrids can infect both fish hosts just as well as the parental lines. This ability may be due to specific traits that facilitate invasion and infection of both host species.

Are hybrid infection rate estimates biased by selfing?

Eggs collected from hybrid worm pairs represent a mix of self-fertilized and hybrid offspring. Up to 76% of the coracidia typed from hybrid sibships were selfed, yet, of the plerocercoids recovered from fish exposed to hybrids, 27 were hybrids and 5 were selfed. Even though more selfed coracidia were presumably taken for copepod infections, hybrid worms were

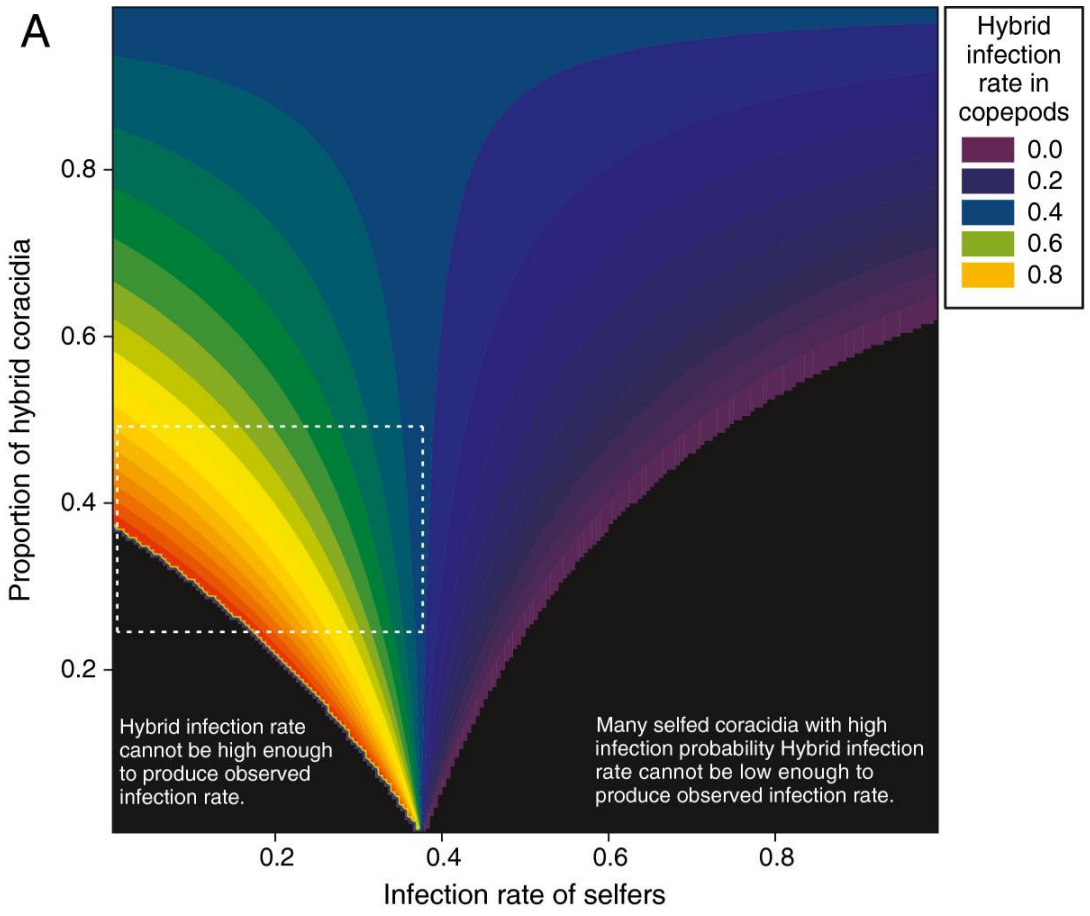
more likely to be recovered from fish at the end of the experiment. This suggests the estimated infection rates of hybrids in copepods and fish may be downwardly-biased; but how much? The observed infection rate in copepods, R_c, equals:

$$R_c = (R_{ch} * P_{ch}) + R_{cs} * (1 - P_{ch}))$$

where R_{ch} is the infection rate of hybrids in copepods, R_{cs} is the infection rate of selfers in copepods, and P_{ch} is the proportion of coracidia that are hybrids. The hybrid infection rate, our primary interest, thus equals:

$$R_{ch} = \frac{R_c - (R_{cs} * (1 - P_{ch}))}{P_{ch}}$$

As R_c is known (=0.375), the infection rate for hybrids can be calculated for different combinations of R_{cs} and P_{ch}. This is shown in Figure II-4A. Inbreeding depression has been observed in *S. solidus* (Christen *et al*., 2002; Schjørring, 2004), so we may expect the infection rate of selfers to be lower than R_c (e.g. for the *S. solidus* population used here, other experiments determined the infection rate of selfed coracidia to be ~10%; D. Benesh, unpublished data). Moreover, the proportion of typed coracidia that were hybrids ranged from 24 to 49%. If we take these values to define a plausible range ($R_{cs} < 0.375$ and $0.24 < P_{ch} < 0.49$), then Figure II-4A indicates that the infection rate of hybrids in copepods may be substantially higher than estimated by the experiment.


A
Hybrid infection rate in copepods
0.0
0.2
0.4
0.6
0.8
Proportion of hybrid coracidia
0.2
0.4
0.6
0.8
Hybrid infection rate cannot be high enough to produce observed infection rate.
Many selfed coracidia with high infection probability Hybrid infection rate cannot be low enough to produce observed infection rate.
0.2
0.4
0.6
0.8
Infection rate of selfers

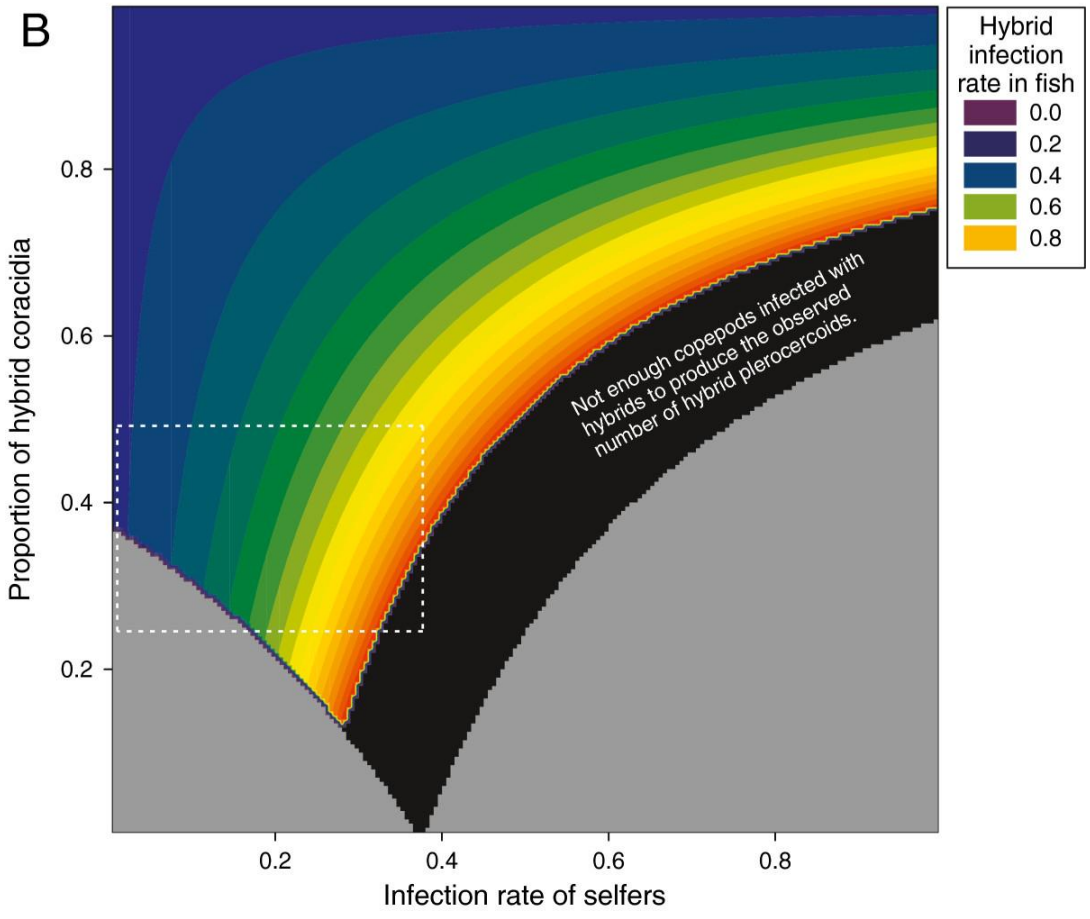

B
Hybrid infection rate in fish
0.0
0.2
0.4
0.6
0.8
Proportion of hybrid coracidia
0.2
0.4
0.6
0.8
Not enough copepods infected with hybrids to produce the observed number of hybrid plerocercoids.
0.2
0.4
0.6
0.8
Infection rate of selfers

Figure II-4: Contour plots of the infection rate of hybrids in copepods (A) and sticklebacks (B) that reproduce the observed data. Contours are plotted as a function of the infection rates of self-fertilized worms in copepods and the proportion of hybrid coracidia. See the main text for the equations used to calculate the hybrid infection rates. Black areas represent parameter space in which the observed results cannot be reproduced; intuitive explanations for this are given in each case. The gray areas in **(B)** are the black areas from **(A)**. Dashed white lines delineate the parameter space that we consider most plausible. The boundaries of this area on the y-axis were based on observed hybridization rates, which ranged from 0.24 to 0.49. The width on the x-axis was based on the assumption that, due to inbreeding depression, the infection rate of selfers is probably lower than the overall mean (0.375).

This approach can be extended to calculate the infection rates in fish necessary to produce the observed number of hybrid plerocercoids. Assuming the proportion of selfed and hybrid worms infecting copepods are the same proportions used for the fish exposure (i.e. there is no differential mortality in copepods between the two groups), then the proportion of fish exposed to hybrid worms, P_{fh}, equals:

$$P_{fh} = \frac{R_{ch} * P_{ch}}{R_c}$$

The number of worms recovered from fish that are hybrids, n_{ih}, is then, $n_{ih} = P_{fh} * n_e * R_{fh}$, where n_e is the number of fish exposed and R_{fh} is the infection rate of the hybrids in fish. Rearranging for R_{fh}**,** our parameter of interest, gives $R_{fh}=n_{ih}/(P_{fh}*n_e)$. n_{ih} and n_e are known (29 and 78, respectively) and P_{fh} is a function of the infection rate of selfers in copepods and the proportion of coracidia that are hybrids. Consequently, we can calculate the hybrid infection rate in fish necessary to produce the observed results, given different initial conditions (R_{cs} and P_{ch}), and this is shown in Figure II-4B. In the parameter space with the highest plausibility, hybrid infection rates were upwardly biased, but only slightly. Only with quite high selfer infection rates (>0.25) does this bias become large enough to suggest that hybrids have significantly higher infection rates in fish than the pure lines (>0.6).

In summary, our calculations indicate that the infection rate for hybrid worms in copepods may be much higher than estimated by the experiment, perhaps even higher than the pure *S. solidus* group (Figure II-2). On the other hand, infection rate estimates in fish do not appear to be so biased that the rate for hybrids should be considered larger than that of the pure lines in their normal host (Figure II-3). Thus, this analysis underscores our main conclusion; the hybrids do not experience any obvious fitness disadvantages compared to pure lines.

Relationship between worm and fish body size

There was a significant relationship between fish weight and worm weight ($F_{1,39}$ = 104.4, P < 0.001). Moreover, this relationship seemed to depend on the worm group (interaction between fish weight and worm group, $F_{3,33}$ = 8.68, P < 0.001) (Figure II-5). Differences between groups were biggest in large fish, with pure *S. solidus* growing particularly large in *G. aculeatus* (Figure II-5). Unfortunately, there were few data points in the largest fish, making these results tenuous. When we eliminated the data points from the largest fish (>0.7 g), there was no longer a significant interaction ($F_{3,29}$ = 0.09, P = 0.96) nor were there significant differences in the mean weight of hybrids and pure species worms ($F_{3,29}$ = 0.27, P = 0.84). Thus, these results suggest that hybrids and pure lines grow to quite comparable sizes in sticklebacks, although it remains possible that in larger fish worm sizes may diverge between groups.

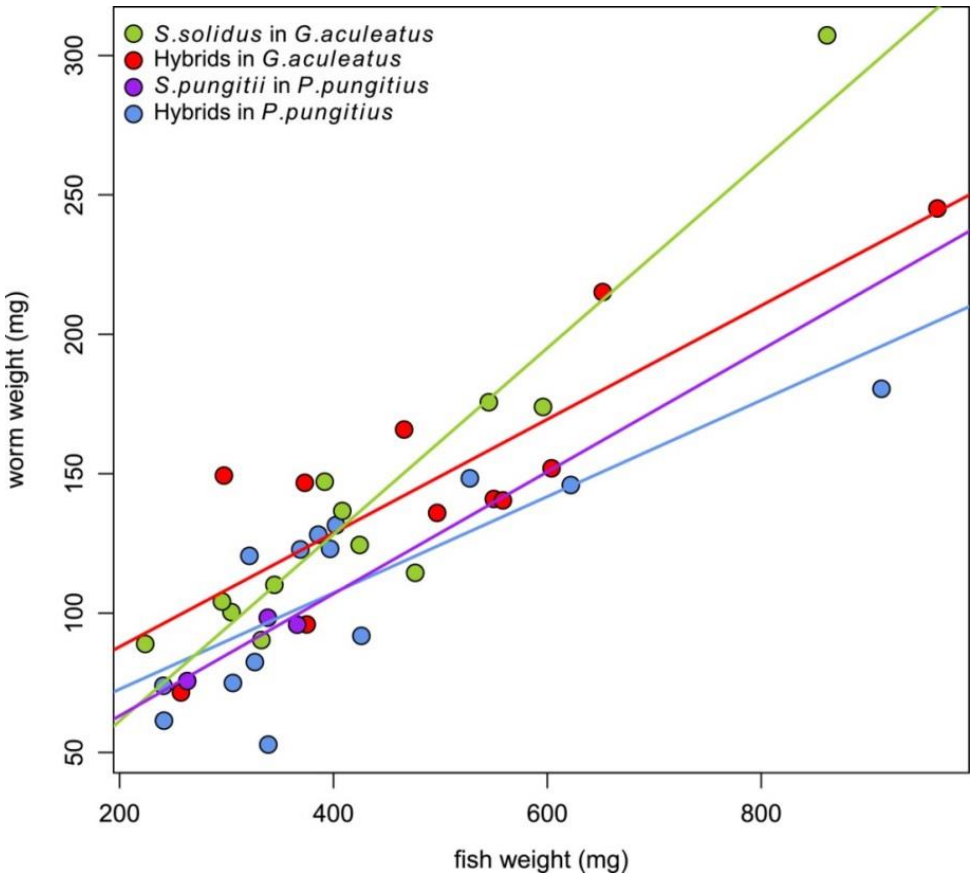


Figure II-5: Relationship between fish weight and worm weight.
The figure shows the relationship of fish and worm weight in mg for the different treatment groups. (*G. ac* with *S. solidus*: n=12, *G. ac* with hybrids: n=11, *P. pu* with *S. pungitii*: n=4, *P. pu* with hybrids: n=14).

Both the size of the host body cavity as well as its immune defenses are likely to limit worm growth. The host's immune system is likely to interact with the parasite and interfere with its growth, as well as the space of the body cavity limits worm growth at a certain point.

Conclusion

What is the evolutionary advantage of being highly host specific and why have no hybrid *Schistocephalus* been found in nature so far? There are several possibilities and none are mutually exclusive.

Ecological factors could cause prezygotic isolation between species. Both parasites species could have completely independent life cycles by inhabiting different microhabitats in the bird's gut or even by infecting different bird species. Although *S. solidus* is known to be infective to a wide range of warm-blooded vertebrates (Dubinina, 1980; Smyth, 1946), much less is known about *S. pungitii*. We also don't know if the relatively high selfing rate observed in hybrid sibships is a consequence of the worms preferring to self instead of hybridizing or a consequence of a high proportion of unviable hybrids that did not hatch.

If the species are separated by postzygotic isolation, it may be that they hybridize frequently, but are either outcompeted by the pure lines or show a F2 hybrid breakdown (Burton *et al*., 2006; Dobzhansky, 1936; Endler, 1977). We could show that at least the F1 generation of hybrids does not show obvious fitness disadvantages, which argues against the fact that they are rapidly outcompeted. Finally, barriers to hybridization may exist only in sympatric populations (reinforcement, for example see Liou & Price, 1994). The *S. solidus* used in this study originate from a population in western Norway, where no nine-spined sticklebacks occur in the whole area (Per J. Jakobsen & Tom Klepaker, personal communication); therefore, in this specific situation there was no selection pressure to evolve a barrier to mating, which might be the case in populations where both stickleback species together with their specific parasites co-occur.

As other studies have shown, hybridization occurs in natural populations of different parasite taxa and has also been shown as a mechanism to broaden the host range by introgression of new genes (Huyse *et al*., 2009; Mutani *et al*., 1985; Pagès *et al*., 2002).

It is worth noting that most studies on *S. solidus* and *S. pungitii* are based on morphological traits, which are not easily distinguishable between the species. To date, only a few studies have employed genetic markers (Andris *et al*., 2012; Nishimura *et al*., 2011) on a limited

number of individuals, and therefore more extensive studies targeting the detection of hybrids are warranted.

Although we could observe hybridization in the laboratory, it still remains unclear if hybridization also occurs in nature. Further experiments are needed to test whether the worms are located in the same compartment of the bird's gut, if hybridization can occur in natural hosts and if given the choice, worms choose mates of the same species over hybridization. Furthermore, it would be interesting to know how the possibility of hybridization and fitness parameters, such as infection rates in intermediate hosts, vary between sympatric and allopatric pairs of *S. solidus* and *S. pungitii* or if there even is a barrier to hybridization in nature. We are currently collecting more species pairs from different populations to test these ideas.

Acknowledgements

We would like to thank R. Leipnitz and M. Schwarz for help with the copepods, G. Augustin and D. Martens for fish husbandry, P. J. Jakobsen for help in collecting Norwegian sticklebacks. We also thank P. J. Jakobsen, I. Samonte-Padilla, K. B. Mobley and P. Rausch for helpful discussions.

Chapter III

Prezygotic barriers to hybridization in *Schistocephalus* spp.?

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Unsubmitted manuscript

Abstract

The cestodes *Schistocephalus solidus* and *S. pungitii* are closely related parasites that each exhibit a strong specificity for their second intermediate host, three- or nine-spined sticklebacks. However, they can share the same final avian host where they reproduce sexually. The two species produce viable hybrid offspring in an *in vitro* culture system, even though molecular data suggests a deep degree of divergence between the two species. In this study, we examined possible prezygotic mechanisms that could prevent hybridization in natural populations. Our results show that the two species share the same microhabitat within their final hosts and do hybridize under natural conditions. We furthermore could find no indications of barriers to hybridization in sympatric populations. Strikingly, a mate choice experiment showed that parasites of the different species were preferred over conspecifics.
In conclusion we could find no mechanism that would effectively prevent natural hybridization in this species. This suggests that postzygotic mechanisms, such as genetic incompatibilities in the F2 generation, may play a major role in the evolution of these two parasite species.

Background

Natural hybridization may play a major role in the evolution of species complexes, but is a controversial topic among evolutionary biologists for some time (Barton, 2008). The major controversy revolves around whether natural hybridization is a beneficial or a disruptive process for speciation. While many botanists argue that hybridization can lead to the formation of new species (e.g. Anderson, 1949; Grant, 1981; Stebbins, 1959), many zoologists see it as a rather disruptive process that reinforces reproductive isolation between species (e.g. Turelli *et al.*, 2001).

The outcome and impact of natural hybridization can vary greatly among species or species complexes and depends largely on the fitness of the hybrids. If hybrids are relatively unfit, hybrid zones are defined as clines maintained by a balance between dispersal and selection against hybrids (Barton & Hewitt, 1985). Hybrids are usually regarded as less fit than their parental species, but can also have a fitness advantage in certain habitats when novel genotypes have the potential for adaptation (Nolte & Tautz, 2010). Cross-species matings can have several outcomes: 1) no viable offspring, 2) F1 offspring that is viable but infertile, 3) F1 offspring which are viable and fertile but outcompeted by parent species (Rundle & Nosil, 2005), or 4) F1 offspring that are viable, fertile and fit enough to spread to unoccupied ecological niches (Arnold & Hodges, 1995; Dobzhansky, 1970; Grant, 1963).

Barriers to hybridization can be divided into pre- and postzygotic barriers. Prezygotic barriers include behavioural and ecological factors that may prevent matings between different species as well as genetic incompatibilities between the gametes. Postzygotic barriers include the potentially lower fitness of hybrid offspring as well as F1 infertility or F2 hybrid breakdown (Dobzhansky, 1970).

Hybridization has been shown in all major parasite taxa, including digeneans (Leroux, 1954, Taylor, 1970, Agatsuma *et al*., 2000; Morgan *et al*., 2003), monogeneans (Kuusela *et al*., 2007; Schelkle *et al*., 2012), nematodes (Grabner *et al*., 2012; Martín-Sánchez *et al*., 2005) and cestodes (Okamoto *et al*., 2010). Functional analyses of hybrids exhibited varying phenotypes. Hybridization in schistosomes (either between two different species or two different strains) has been shown to affect host range (Huyse *et al*., 2009; Mutani *et al*., 1985; Pagès *et al*., 2002), behavioural traits like cercarial shedding patterns (Théron & Combes, 1988) and host-finding behaviour (Kalbe *et al*., 2004). As these traits are of crucial importance for the interaction between parasites and their hosts, hybridization can impact the evolution and epidemics of parasites.

We recently found that hybridization between two *Schistocephalus* species resulted in viable hybrids that were able to expand their host range on the level of the second intermediate hosts and showed only a low level of reduction in overall fitness (Henrich *et al*., 2013). Both parasites occur in sympatry and it is likely that they frequently meet in the final host and could hybridize in natural populations.

Since the two species show a high degree of genetic divergence (Nishimura *et al*., 2011), it can be assumed that selective forces keep the two species separated and prevent gene flow. Therefore, we test different possibilities for barriers that could prevent hybridization in natural populations of these species. We experimentally test if there are i) spatial constraints to hybridization (i.e. are the two species localized in the same area of the gut of a final host or do they use different compartments?), if ii) there are barriers to hybridization in sympatric populations or if iii) there is assortative mate choice by species.

To test these questions, we conducted experimental infections of chickens (*Gallus gallus*), herring gulls (*Larus argentatus*) and a mate choice experiment using an artificial breeding system.

Methods

Study organisms

Schistocephalus solidus and *S. pungitii* are closely related cestodes with a complex life cycle involving three different hosts (Clarke, 1954; Dubinina, 1980). Both species use piscivorous birds as final hosts, where the adult worms reproduce sexually. These parasites are simultaneous hermaphrodites, mature rapidly and usually complete the reproduction within one week in the final host (Dubinina, 1980; Schärer & Wedekind, 1999). The eggs are then released with the bird's feces into the water, where they hatch into free-swimming larvae. These larvae have to be eaten by cyclopoid copepods, the first intermediate hosts, to develop into procercoids. If infected copepods are eaten by sticklebacks, the second intermediate hosts, the parasite migrates through the gut wall into the fish's body cavity and develops into a plerocercoid. Both parasites are highly specific on this level of the life cycle: *S. solidus* can only infect three-spined sticklebacks (*Gasterosteus acueleatus*), while *S. pungitii* is only infective to nine-spined sticklebacks (*Pungitius pungitius*). Several experiments have already investigated this phenomenon of high host specificity (Bråten, 1966; Orr *et al*., 1969, Henrich & Kalbe in prep.) and hybrids of the two parasites species show an expanded host range and

could infect both stickleback species (Henrich *et al*., 2013). The life cycle is completed when piscivorous birds prey upon infected sticklebacks.

The life cycle for both cestodes can be completed in the lab by replacing the final host with an artificial breeding system (Smyth, 1946; Wedekind, 1997). For this purpose, worms are placed into sealed net bags and incubated in a 40°C warm culture medium (for details see Wedekind, 1997) in the dark for a time period of up to 8 days, where most of the egg production is accomplished (Dubinina, 1980; Schärer & Wedekind, 1999). The eggs are then washed and stored in tap water at 4°C before development is induced. Hatching can be triggered and mostly synchronized (by exposure to light), which facilitates experimental exposure of both copepods and later on fish.

Spatial constraints

To test whether spatial constraints inhibit either parasite from hybridization, we investigated where the parasites are located in the gut of the final host. For this purpose we used plerocercoids from wild caught sticklebacks to expose chickens (*Gallus gallus*). *G. gallus* was previously used as an experimental final host by Tierney & Crompton (1992). Nine-spined sticklebacks were caught in Lebrader Teiche, Germany (54° 22’ N, 10° 42’ E) and three-spined sticklebacks in lake Skogseidvatnet, Norway (60° 14’ N, 05° 55’ E). These are the same parasite populations as used in Henrich *et al*. (2013), from which we knew they hybridized in the artificial breeding system. The fish were killed with an overdose of MS222 (tricaine methanesulfonate, 1mg/ml) followed by a cervical incision, and the plerocercoids removed and weighed. To avoid selfing, we size-matched the plerocercoids by body weight (Lüscher & Milinski, 2003) before placing them into the empty body cavity of uninfected sticklebacks.

Each of the three chickens was fed 16 plerocercoids (8 *S. solidus* and 8 *S. pungitii*). In order to decrease the chance of damage to the parasite by the chicken through gizzard stones we fed the chickens with soft cat food for two days prior to parasite exposure. After 48 hours the chickens were killed and the gut was removed. The dissection of the gut was performed to collect the parasites and identify their location.

Sympatric vs. allopatric hybridization

Two herring gulls (*Larus argentatus*) were infected with 8 plerocercoids each (4 *S. solidus* and 4 *S. pungitii*). One gull was exposed to 8 cestodes of the two species that both originated from a sympatric population in Obbola, Sweden (63° 39’ N, 20° 17’ E). The second gull was

exposed to 4 lab-bred cestodes of *S. solidus* from a population in Skogseidvatnet, Norway and 4 cestodes of *S. pungitii* from wild caught nine-spined sticklebacks from Lebrader Teiche, Germany. The aim of this experiment was to test, if different allopatric or sympatric species hybridize under natural conditions. There is no population of nine-spined sticklebacks that could harbor *S. pungitii* in the vicinity to the population of three-spined sticklebacks from Skogseidvatnet, Norway. Consequently this *S. solidus* population is very unlikely to encounter *S. pungitii* frequently and need not develop a barrier to hybridization, whereas mating barriers are more likely to arise in places where both parasite species occur in sympatry.

Feces of the herring gulls were collected 24, 48 and 72 hours after infection. Eggs were washed and incubated at 20°C in the dark for three weeks before coracidia were hatched. For each time point, 32 coracidia were collected and analyzed. In summary 96 coracidia per gull were typed using microsatellite markers (Binz *et al*., 2000).

Mate choice experiment

S. pungitii plerocercoids originated from field-collected *P. pungitius* caught at Lebrader Teiche, Germany. *S. solidus* plerocercoids were obtained from lab-infected *G. aculeatus* (12 weeks post exposure) and those originated either from a population from lake Skogseidvatner, Norway or Xinzo de Limia, Spain (42° 07’ N, 07° 39’ W). We chose *S. solidus* cestodes from two different populations to ensure that those plerocercoids were derived from different families. As it has been shown earlier that *S. solidus* prefers closely related over distantly related mates (Schjørring & Jäger, 2007), we wanted to ensure that this does not affect our experiment. We assumed that the likelihood of two *S. pungitii* from a wild caught population being closely related was rather low.

The sticklebacks were killed with an overdose of MS222 (tricaine methanesulfonate, 1mg/ml) followed by a cervical incision. Afterwards the plerocercoids were removed from the fish, and weighed to the nearest 0.01mg. We size-matched the plerocercoids in all experimental triplets to avoid parasite size as a factor in mate choice, as *S. solidus* has been shown to prefer bigger mates (Lüscher & Wedekind, 2002). The parasites were then placed in fork-shaped nylon mesh bags in a randomized order. This experimental setup was previously used and is described in further detail in Lüscher & Wedekind (2002). Briefly, the mesh bags consisted of three compartments separated by seams (Figure III-1). The focal worm was placed in the middle prong while the stimulus worms were placed in the two side prongs. All three openings of the bags were closed by melting the nylon ends with a flame. Each bag was then

placed in a glass container filled with culture medium (Smyth, 1946; Wedekind, 1997) pre-warmed to 40°C and covered with a lid to avoid evaporation. At the start of the experiment, the containers were placed in an incubator equipped with weak red light and a camera set and recording was started 15mins after the container was placed in the experimental chamber.
Mate choice trials ran for 2800min, and a picture was taken once every minute. In summary we recorded and evaluated 14 mate choice trials (7 for *S. pungitii* and 7 with *S. solidus* as the focal worm) and 5 control trials with just two worms (one as a stimulus worm and the other as a focal worm, always of the same species).

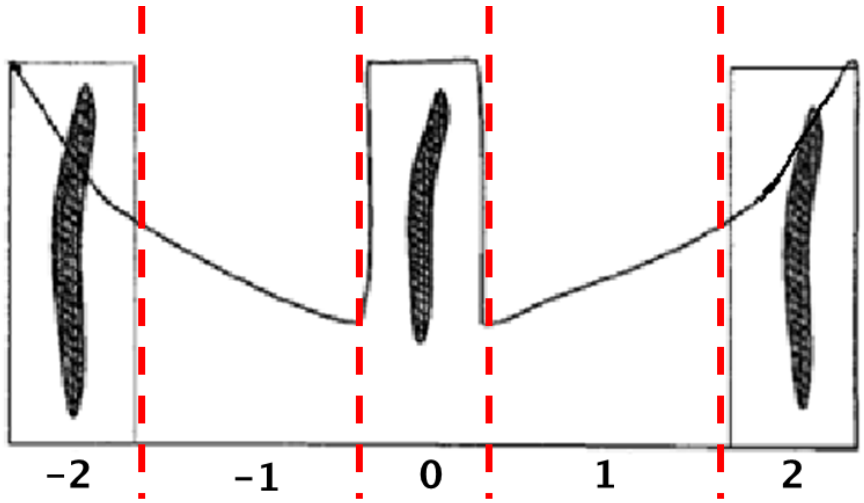

Figure III-1: Setup for mate choice experiment (modified after Lüscher & Wedekind, 2002)
A focal worm was placed together with two stimulus worms into a fork-shaped mesh bag in a culture medium mimicking the situation in the bird's gut. The focal worm (middle compartment) can move freely between all compartments, while the stimulus worms are restricted to side compartments that overlap the middle compartment. Scores were assigned according to the focal worm's position. "0" was considered neutral, 1 & 2 (or -1 & -2 respectively) for a tendency to the side of one of the stimulus worms, and if there was a minimum of 25% overlap between the focal worm and one of the stimulus worms, we assigned the score "3" or "-3", as this was a position where mating was possible.

While the stimulus worms could not leave their compartment, the focal worm could position itself anywhere. As the decisive criteria, the largest part of the focal worm`s body (>50%) was assigned to one of the 5 positions at each minute (-2, -1, 0, 1 or 2, Figure III-1), or, if more than 25% of two worms overlapped, this was counted as a possible mating attempt and classified with "-3" or "3". The scoring of all positions was carried out by one blind observer regarding the experimental setup. Only after all scores were assigned and all failed trials (in

some cases the plerocercoids managed to escape from the mesh bags) were excluded, did we assign the position to the parasite identity. We then classified all positive scores (1, 2 or 3) to the side with the conspecific and all negative scores to the side with the parasite from the different species (-1, -2 and -3).

Results

Temporal constraints

Infection of chicken showed that the worms of both species settled in the appendices of the chicken gut. From a total of 24 worms used in this experiment, only three could be recovered 48hours after exposure from two chickens. We have no data on how many worms successfully established in the herring gulls because the birds were not dissected due to animal welfare regulations. However, we could collect eggs from the feces of the infected herring gulls and hatch coracidia. Microsatellite typing indicated that there was hybridization in the herring gulls.

Barriers to hybridization in sympatric populations?

We tested for hybridization in herring gulls and both sympatric and allopatric combinations of worms showed hybridization in the natural host (Figure III-2). The distribution of offspring (the proportion of S. *solidus*, *S. pungitii* or hybrids) differed between the two gulls ($\chi^2 = 13.59$, df = 2, $p < 0.005$). The hybridization rate was lower in the sea gull that was infected with a sympatric combination. Despite the small sample size we can say that it seems unlikely, that sympatric populations evolved a strong barrier to hybridization.

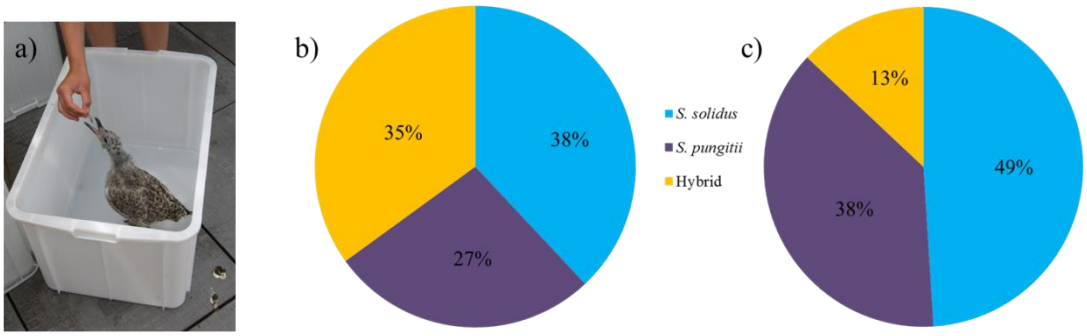

Figure III-2: Hybridization of *S. solidus* and *S. pungitii* in their natural host. (a) Infection of lab-reared herring gulls and distribution of species in (b) allopatric and (c) sympatric combinations, determined by microsatellite typing of tapeworm larvae hatched from eggs isolated from the gull droppings.

Mate choice

When we analyzed our controls to see if worms actually did chose a worm over an empty compartment, we saw significant differences between the three assigned sides (worm, neutral, nothing) (ANOVA, $F_{2,15} = 8.009$, $p < 0.05$). Worms spent significantly more time on the side with another worm (in this case always a conspecific), than in the neutral or empty compartment (post hoc Tukey HSD, $p < 0.05$).

When the data for both focal worm species was combined, there was a significant difference in time spent in each compartment (ANOVA, $F_{2,41} = 6.125$, $p < 0.05$). A post hoc Tukey HSD test showed that the focal worms spent more time in the compartment with a different worm species than with the conspecific ($p < 0.05$), but also more time in the neutral zone than with a conspecific ($p < 0.05$, Figure III-3).

However, analyzing the data for each species separately, only one of the species displayed a significant preference: *S. pungitii* focal worms showed a significant difference in time spend on each side (ANOVA, $F_{2,20} = 4.923$, $p < 0.05$). A post hoc Tukey HSD test showed that *S. pungitii* spent significantly more time with a worm from a different species than with a conspecific ($p < 0.05$). There was no significant difference between time spent in the neutral zone and any side with a stimulus worm in *S. pungitii*. Even though *S. solidus* worms also spent more time on the side with a worm of a different species than on the side with a conspecific, the difference was not significant (ANOVA, $F_{2,20} = 2.063$, $p = 0.156$).

Taken together, our results suggest that focal worms spent significantly more time in a possible mating position (-3 or 3 respectively) with a worm from a different species, than with a conspecific (T-Test, $t = -2.069$, $df = 26$, $p < 0.05$). However, if the two species were analyzed separately, the effects were not as strong. Both *S. pungitii* (T-Test, $t=-0.888$, $df = 12$, $p = 0.392$) and *S. solidus* (T-Test, $t = -1.874$, $df = 12$, $p = 0.086$) do not show a significant difference in time spent in a possible mating position with either partner. Nevertheless, *S. pungitii* spent 7.79% (±2.60%) of the total time in a possible mating position with a conspecific and 10.92% (±2.37%) with the worm from a different species. *S. solidus* spent 5.88% (±2.90%) of the total time in a possible mating position with a conspecific and 18.21% (±5.90%) with the worm from a different species.

The detailed profiles of each focal worm's location over time can be found in the supplementary material (SI Figure S1).

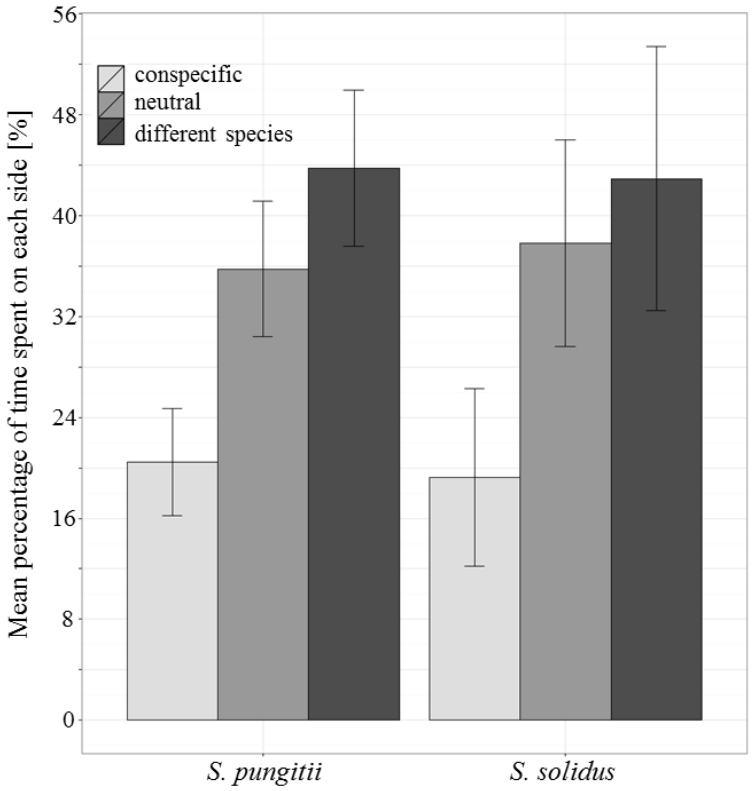

Figure III-3: Mean percentage of time spent in the different compartments

For each focal worm species (*S. pungitii* or *S. solidus*) the mean percentage of time on each side (conspecific: 1, 2 or 3, neutral: 0, different species: -1,-2 or -3) was calculated for each trial and the summarized means (±SE) are shown. On average, both focal worm species spent more time on the side that contained a worm from a different species than with a conspecific or in the neutral compartment.

Discussion

The results from our experiments indicate that neither temporal nor spatial constraints hamper the two *Schistocephalus* species from mating in the final host. Both worm species establish in the same part of bird's intestines. Even though the majority of plerocercoids did not establish in the chicken host, we nevertheless found plerocercoids from both species in the chicken appendices. This indicates that either the adult chicken is not a suitable final host or other factors prevented successful establishment of the plerocercoids. It has been shown earlier that *S. solidus* is infective to day-old chicken (Tierney & Crompton, 1992), but the digestive tract, especially the structure of the gizzard, may have lowered the infectivity in our experiment. Birds that belong to the order Galliformes have relatively long appendices, similar to birds that belong to the Anseriformes (which also include a number of piscivorous birds), but can

be totally absent in some birds like cranes (Storch & Welsch, 2009). Since *Schistocephalus* can probably infect any fish-eating bird, the exact location may vary across host species and depend on the anatomy of the final host species.

Nevertheless, the experiment with seagulls showed that both species do mate with each other in a natural final host. How likely is it then, that a bird feeds on both infected stickleback species? Birds feeding on infected sticklebacks are unlikely to discriminate between *G. aculeatus* and *P. pungitius*. Even though both stickleback species inhabit slightly different ecological niches (with *P. pungitius* preferring more vegetated areas and *G. aculeatus* roaming also more in the open water (Coad & Power, 1973a, 1973b)), there is still an overlap in both species' habitat and they are often caught together within the same shoal (Hynes, 1950; Zander *et al*., 1984).

For example, in one population we used in this study from Obbola (Sweden), both stickleback species were caught with the same method (seine fishing) in the same net. Therefore it is very likely that piscivorous birds prey on both stickleback species at the same time. Since the infection rate for *S. solidus* and *S. pungitii* in this population is very high for both species (70-90%, unpublished data), we consider the likelihood of the two species ending up in the same final host relatively high.

Our results also indicate no obvious barriers to hybridization in sympatric populations of *Schistocephalus*. Even though the hybridization rate in the herring gull infected with a sympatric combination of plerocercoids was slightly lower, we could detect hybrid offspring.

It has been shown, that in some cases prezygotic barriers can arise in sympatric populations that prevent the formation of viable hybrids (e.g. reviewed in Rundle & Nosil, 2005). The mechanism by which prezygotic barriers arise can be a by-product of divergence (Coyne & Orr, 2004; Rice & Hostert, 1993; Schluter, 2001) or hybridization can occur frequently, but hybrids are relatively unfit compared to their parental lines, and therefore outcompeted, which leads to reinforcement (Servedio & Noor, 2003). Whether the hybrids from sympatric combinations have a lower fitness than hybrids from allopatric combinations was not tested in this experiment. Such an experiment should be conducted under controlled laboratory conditions and should, if possible, include different sympatric population pairs.

As we have shown in earlier experiments (Henrich *et al*., 2013), hybrids of both species (from allopatric populations) do not suffer from obvious fitness disadvantages, but rather have the advantage of increasing their host range without the cost of reduced performance in the intermediate host. At least in these hybrids formed in allopatric combinations, fitness

disadvantages occur only after hatching (which seemed to be the only trait where hybrids were less fit than the parental species).

It is rather surprising that our results indicate a preference of both parasite species to mate with a parasite of a different species over conspecifics. It has been shown that *S. solidus* prefers to mate with siblings over a more distantly related conspecific (Schjørring & Jäger, 2007). Our results point in a completely different direction, indicating that the parasites might prefer maximal genetic distance in their mating partners, not taking species boundaries into account.

From our controls we can also conclude that the time spent on the side with a mating partner is representative, as parasites spent significantly more time on the side with a mating partner than in "empty" compartments. *Schistocephalus* is a simultaneous hermaphrodite that can engage in multiple matings during its reproduction period. Our system allowed multiple mating attempts and most parasites visited both stimulus worms during our trial (see Figure S1). However, we conclude that the total percentage of the time spend on one side is a good indicator for mate choice. This has also been previously shown by Lüscher & Wedekind (2002) who demonstrated that *S. solidus* discriminates between sizes in their mating partners, preferring bigger mates.

In summary, we did not find any prezygotic mechanisms that could prevent hybridization between *S. solidus* and *S. pungitii*. It is possible that hybridization occurs frequently in some populations but has not been detected yet. Nevertheless, it seems obvious that the high degree of host specificity in this system was a major factor driving the separation and speciation of these two parasite species.

In general, there are two different possibilities for speciation through host specificity in parasites (Brooks & McLennan, 1993; Shaw, 1994; Thompson, 1994): either through host-switching or through congruent co-speciation. Host-switching requires an initial decrease in host specificity for the parasite in order to be able to establish in the new host, followed by a compulsory subsequent increase in host specificity, that is necessary to discriminate between host range expansion and host switching. Another mechanism for hybrid speciation is following the speciation of the host lineages, where parasites and hosts exhibit congruent phylogenies, a process which is described as Fahrenholz's rule (Eichler, 1948). We don't know how speciation occurred in *Schistocephalus*. Nishimura *et al*. 2011 suggested that speciation occurred shortly after the divergence of the two stickleback lineages as a single event, which would argue for a co-speciation following the divergence of the stickleback lineages.

Studies on gene flow between different species or populations of *Schistocephalus* are still rare (Nishimura *et al*., 2011) and should also consider the possible gene flow between these two species in the future. Even though the research conducted to date indicates that separation of the two lineages occurred 20-25mybp, the situation in other populations may differ from this assumption. If speciation in *Schistocephalus* is not a recent event and the two species now differ by a relatively large extend, postzygotic isolation seems plausible. The more loci differ between two species, the more it is likely that negative interactions between them evoke or strengthen postzygotic isolation (Coyne & Orr, 1998).

It is possible that gene flow in natural populations of *Schistocephalus* is limited by postzygotic barriers. So far we do not know if *Schistocephalus* hybrids are sterile or if other genetic incompatibilities would lead to an F2 hybrid breakdown. This point warrants further investigation.

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Chapter IV

Intraspecific competition alters the virulence of the parasite *Schistocephalus solidus* in three-spined stickleback

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Unsubmitted manuscript

Abstract

Parasites often share the same host with conspecifics. Conflicts can arise when parasites compete for nutrients or use different strategies for manipulation of behavior or immune functions of their hosts. These interactions can directly alter growth, survival and transmission of the parasites, but also indirectly influence the host's fitness.

The tapeworm *Schistocephalus solidus* is a parasite with a complex, three-host life cycle. It spends the majority of its life and completes its entire somatic growth in the second intermediate host, the three-spined stickleback (*Gasterosteus aculeatus*). Since it spends most of its life cycle in this host, it is likely that interactions between the parasite and its host have been fine-tuned through evolutionary time.

The close co-adaptation of *S. solidus* to its stickleback host offers an ideal system to study intraspecific parasite-parasite interactions and their outcome for both parasite virulence and host fitness. In an experimental approach we compared the competitive situation in double infections with *S. solidus* from two different strains that differ in their virulence.

For this purpose we exposed lab-bred sticklebacks simultaneously to either two high virulent (Hv) or two low virulent (Lv) *S. solidus* tapeworms, or to a combination of both, along with single exposed and unexposed control fish.

Our results indicate complex parasite-parasite(-host)-interactions: slow growing Lv parasites benefit from co-infections with fast growing Hv parasites by an increased virulence while the Hv parasite stays smaller than in the presence of another highly virulent conspecific.

Introduction

In ecology and evolutionary biology the term virulence is used to describe various aspects of host-parasite/pathogen interactions, ranging from parasite induced host mortality (Ebert & Herre, 1996; Frank, 1996) or parasite induced reductions in the reproductive success over the host's lifetime (Herre, 1993) to the general level of harmfulness caused by a parasite (Ewald, 1995).

While natural selection will select for a higher resistance in the host, depending on the mode of transmission it will not always favor the most virulent parasites (Ewald, 1993). Parasites experience a trade-off between high virulence and host overexploitation – therefore parasite fitness will probably be maximized at an intermediate virulence level (Anderson & May, 1982; Fenner & Ratcliffe, 1965). The "virulence-transmission" trade-off may especially affect parasites with complex life cycles as excessive damage to their intermediate host can cause a decrease in transmission success to the next host.

Parasites that significantly damage their hosts evoke selection against that particular parasite or genotype in the host population. A higher virulence that causes greater damage results in a stronger selection against that parasite in the host and favors more resistant host strains. This inevitably leads to a co-evolutionary arms race known as Red Queen dynamics (Van Valen, 1973).

In natural ecosystems, an organism is rarely infected by only one parasite (Petney & Andrews, 1998). In fact, a host usually harbors multiple parasites that often belong to different species or even genera. Conflicts can arise when parasites compete for host resources, manipulation of the immune system or manipulation of the host's behavior and studies on such topics should consider multiple infections more often (Milinski, 2014). These conflicts can be grouped into two categories: interspecific and intraspecific parasite competition. In the case of intraspecific competition, the degree of relatedness between the parasites can alter the outcome (Bashey *et al.*, 2007; Frank, 1992; Jäger & Schjørring, 2006). It is also possible that the infection with one parasite prevents or hampers the infection with another individual from the same species (concomitant immunity, (Smithers & Terry, 1969)). Furthermore, it has been shown, that infection with multiple parasite genotypes can be costly for the host's immune response and so be more detrimental to the host (Taylor *et al.*, 1998). Competition can be a selection factor in the evolution of parasites and promote competitive strategies or phenotypic plasticity (Leggett *et al*., 2014; Mideo, 2009). It can directly lower the individual parasite's fitness or decrease the transmission success to the next host.

Conversely it can result in a fitness advantage for the parasite, where within-host competition favors virulence, with more virulent strains having a competitive advantage in genetically diverse infections (de Roode *et al*., 2005), leading to an increase in virulence in the next generation of parasites. In fact, it has been shown in an experiment with rodent malaria (de Roode *et al*., 2005) that there is a strong relationship between the virulence of a parasite and its competitive ability: the more virulent strains had a competitive advantage in mixed-strain infections which led to a higher relative transmission success. Therefore, within-host competition can drive the evolution of virulence and could be one explanation as to why many parasites harm their hosts.

Other empirical studies have shown that less virulent strains can be favored in competitive situations, which can drive evolution towards lower virulence (Gower & Webster, 2005). Therefore the consequences of competition will depend on the type of interaction among the parasites, the life cycle of the parasite and the degree of interaction with their host and each other. Most microparasites multiply within their host, allowing for within-host selection and adaptation to other co-infecting parasite genotypes. Parasites that do not multiply within the host may face a different situation. They might compete for host resources, space or host manipulation, and interfere with their coinfecting competitors (Cézilly *et al*., 2014).

Most studies about within host competition are observational and analyze macro- and microparasite communities in naturally infected hosts (Fenner & Ratcliffe, 1965; Herre, 1993; Mideo, 2009). In an experimental approach, we investigated the competitive situation between two individual macroparasites of the same age and species infecting a naive host. We were especially interested in how parasites with different intrinsic levels of virulence performed when competing against each other. Does it alter their ability to successfully infect a host and the plasticity of their individual virulence?

If virulence is solely determined by intrinsic factors of the parasite, one would expect virulence to be independent of the virulence of the co-infecting parasites. In the case of multiple infections each parasite may produce resources that would be collectively available (public goods) among conspecifics (Leggett *et al*., 2014). These public goods could be anything that can be used by both parasites, e.g. something that increases the availability of nutrients or protection from the host's immune system. One could also assume that an individual's production share of public goods is proportional to the virulence of the parasite.

We can categorize the public goods into unspecific or specific public goods. Unspecific public goods can be available to all conspecifics, while specific public goods are only available to parasites of a certain genotype, strain or population.

In the case of the production of public goods, the observed virulence would then be dependent on the virulence of co-infecting parasite (SI Figure S2).

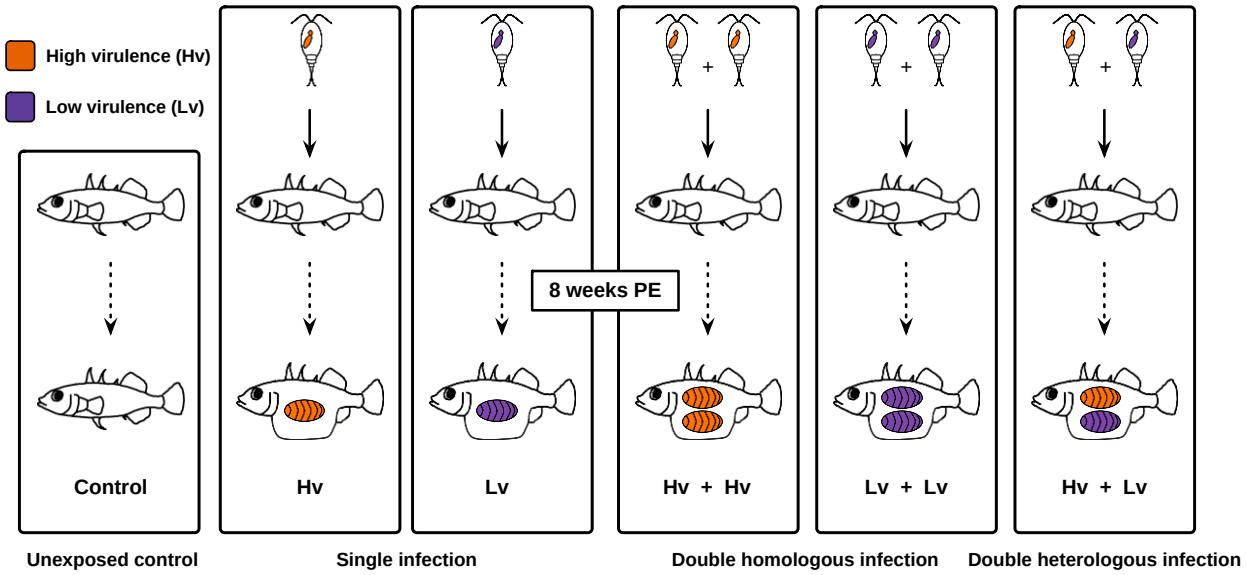

Figure IV-1: Experimental Design

Fish were exposed to one copepod infected with a Hv or Lv parasite, two copepods of either virulence (Hv+Hv and Lv+Lv) or two copepods with parasites of different virulence (Hv +Lv). One group was unexposed control fish. Fish were dissected eight weeks post exposure (PE).

To explore the consequences of multiple infections on individual parasite virulence, we compared the performance and phenotype of single macroparasites under intraspecific competition in an experimental setup with double infections with high virulent (Hv) and low virulent (Lv) parasites. Our model system to explore this question was *Schistocephalus solidus*, a trophically transmitted cestode with a complex life cycle (see methods) and its second intermediate host, the three-spined stickleback (*Gasterosteus aculeatus*). This system is an emerging model in host-parasite coevolution and speciation genetics (Barber & Scharsack, 2010; Barber, 2013; Gibson, 2005; Hammerschmidt & Kurtz, 2005a). This particular stage is important because the parasite establishes in the body cavity, completes the majority of its growth and thus requires a long term interaction with the host immune system. We generated six different treatment groups: double infections with a heterologous combination of parasites (Hv+Lv), double infections with a homologous parasite combination (Hv+Hv and Lv+Lv), single infections with either parasite (Hv and Lv), as well as one unexposed control group (Figure IV-1). Parasite performance was measured as parasite growth, which is an indicator not only for virulence but also for parasite fitness in this

system. Several fitness relevant organ measurements of the fish host served as an indicator for host's response to parasitic infection.

Results

Infection rates

Neither in singly exposed fish (Lv or Hv, $F_{1,548} = 0.313$, $p = 0.31$) nor in fish exposed to two parasites (Lv+Lv, Hv+Hv or Hv+Lv, $F_{1,929} = 0.538$, $p = 0.58$) could we detect an effect of the treatment on the infection rate. There was also no significant difference in the number of successfully established parasites (0, 1 or 2) in the fish exposed to two parasites ($F_{2,929} = 0.049$, $p = 0.95$). The probability of successful parasite establishment in the fish therefore did not differ between Lv and Hv parasites, neither in single nor in double exposure treatments indicating no direct link between parasite infectivity and virulence in this system.

Total parasite index

As a measurement for the total parasite burden, the total parasite index (tPI) describes the relationship of total parasite weight and the fish weight (Figure IV-2).

The tPI differs significantly between treatment groups ($F_{4,321} = 354.502$, $p < 0.0001$). All treatment groups differ significantly from each other (Tukey HSD, $p < 0.01$), except for the comparison of single and double infections of low virulence parasites (Lv vs. Lv+Lv, Tukey HSD, $p = 0.88$). Parasites from a Hv+Hv double infection had the highest tPI, followed by Hv single infections. The heterologous Hv+Lv double-infection had an intermediate tPI. Lv parasites had the lowest tPI, with the double infection having a higher total parasite weight than the single infections.

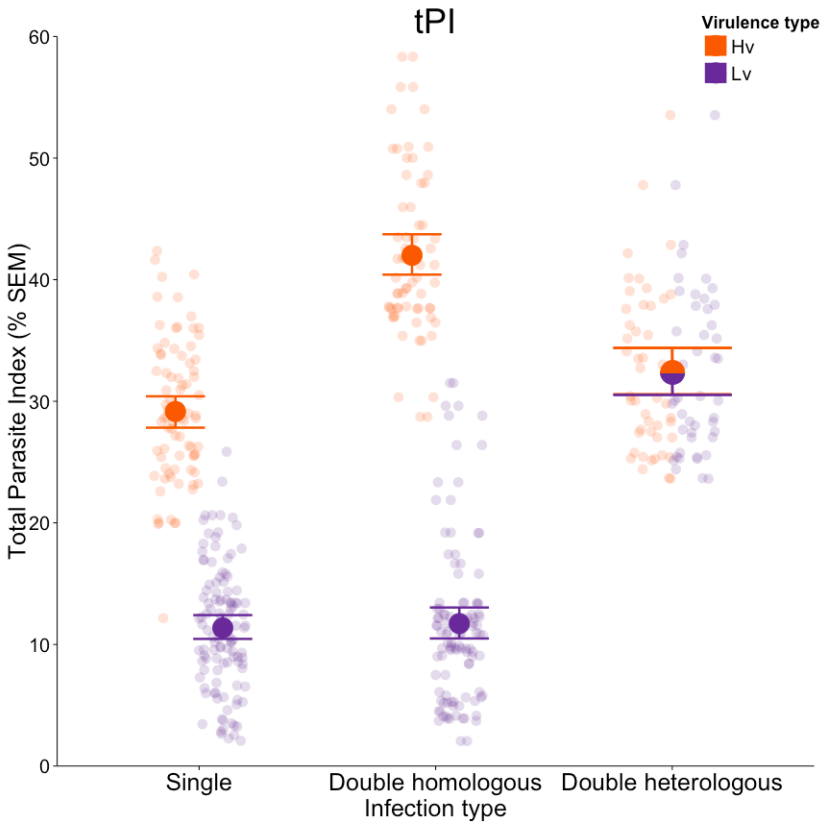

Figure IV-2: Total parasite index (tPI) in the different treatment groups

The homologous double infection of two high virulent parasites (Hv+Hv, N = 33) had the highest tPI, followed by the heterologous double infection (Hv+Lv, N = 47) and single infections by high virulent parasites (Hv, N = 80). Single and double homologous infections with low virulent parasites (Lv, N = 113 and Lv+Lv, N = 53) had the lowest tPI and were not significantly different from each other. Error bars represent standard errors of the mean.

Virulence/discrete parasite index

For this analysis the relative weight of the individual parasite (dPI), was compared between the different treatment groups (Figure IV-3). There was a significant effect of a term which combined the treatment group and parasite virulence type and therefore discriminated between Lv and Hv parasites in the "Hv+Lv" treatment in double infected fish ($F_{5,453}$ = 325.807, $p < 0.0001$). A post hoc Tukey HSD test showed that all groups differ significantly from each other ($p < 0.01$).

The dPI of single infections (Hv and Lv) was significantly higher than the dPI in the respective homologous double infections (Hv+Hv and Lv+Lv). Hv parasites that were in single infections had the highest dPi, followed by Hv parasites in Hv+Hv double infections.

Interestingly, the Hv parasite in the heterologous double infection had a significantly higher dPI than its Lv co-infecting competitor, but significantly lower than in a Hv single infection, or even in a Hv homologous double infection. On the other hand, the Lv parasite in heterologous infection had a significantly higher dPI than in a Lv homologous double infection, or even a Lv single infection.

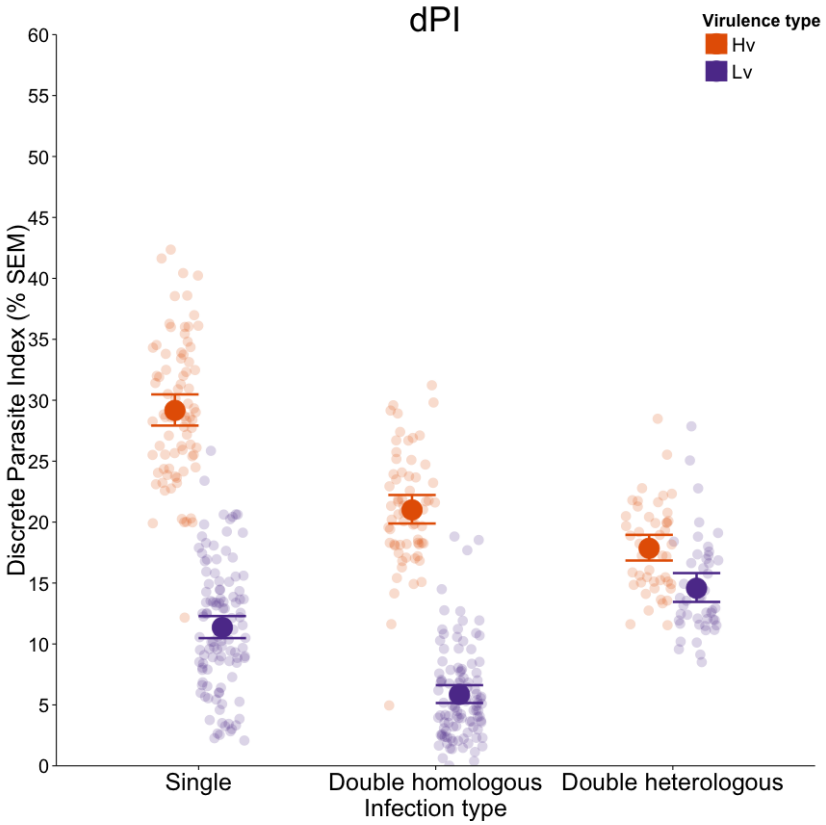

Figure IV-3: Virulence of individual parasites as mean discrete parasite index (dPI) in the different treatments.

The relative worm weight of each individual parasite was altered by intraspecific interactions with co-infecting parasites. Both, high virulent (Hv) and low virulent (Lv) parasites' relative worm weight decreased in double infections with a parasite of the same virulence level (Hv+Hv and Lv+Lv respectively) compared to single infections (Hv and Lv). In mixed infections (Hv+Lv), the more virulent parasite could not reach the same size as in the homologous double infection, while the low virulence parasite grew to an even bigger size than in a single infection. Error bars represent standard errors of the mean (Hv: N = 80, Hv+Hv: N = 66, Lv: N = 113, Lv+Lv: N = 106, Hv+Lv: N = 47).

In summary, when a Hv parasite was in competition with a Lv, the Hv parasite was growing to a significantly smaller size than if it had been in competition with a homologue, whereas

the Lv parasite was clearly gaining from sharing its host with a more virulent parasite, growing significantly bigger than if it has had no competitor.

Fish condition and immunological traits

As an indication for the fish condition, the CF is the ratio between fish length and fish weight, the higher the CF, the better the fish condition.

There was a significant effect of the experimental treatment on the fish condition factor (CF) ($F_{5,392}$ = 15.178, $p < 0.0001$). Only fish exclusively infected with Hv parasite(s) differed significantly from the control fish in their CF (Figure IV-4A). A *post hoc* Tukey HSD test for the different treatment groups showed significant lower CF for fish infected with 1 or 2 Hv parasites compared to the uninfected control fish ($p < 0.0001$). Fish infected with 2 Hv parasites had the lowest CF compared to all other treatments ($p < 0.001$). Fish infected with 1 Hv parasite had a significantly lower CF compared to fish infected with 2 Lv parasites ($p < 0.01$).

The hepatosomatic index (HSI) describes the ratio of fish liver weight to body weight and gives an indication about the energy reserves of the fish (Chellappa & Huntingford, 1995). The higher the HSI is, the more energy reserves the fish has.

There was a significant effect of the experimental treatment on the HSI ($F_{5,392}$ = 17.961, $p < 0.0001$) and a *post hoc* Tukey HSD test showed significant differences between certain treatment groups ($p < 0.001$): Fish from all the infection treatments (Lv, Hv,Lv+Lv, Hv+Hv, Hv+Lv) had a significantly lower HSI than the unexposed control fish (Figure IV-4B). Even in fish singly infected with one Lv parasite, the HSI was significantly decreased, even though the parasite burden is the lowest of all infection treatments. The HSI of homologous single or double infections (respectively, Hv and Hv+Hv, or Lv and Lv+Lv) were not significantly different from each other or from the heterologous double infection (Hv+Lv).

The HSI of Hv single infections was significantly lower than the HSI of Lv single or Lv+Lv double infections ($p<0.05$).

The splenosomatic index (SSI) describes the relationship between the fish's spleen and body weight and elevations in the SSI can be attributed to an immune response to parasite infections (Lefebvre & Mounaix, 2004).

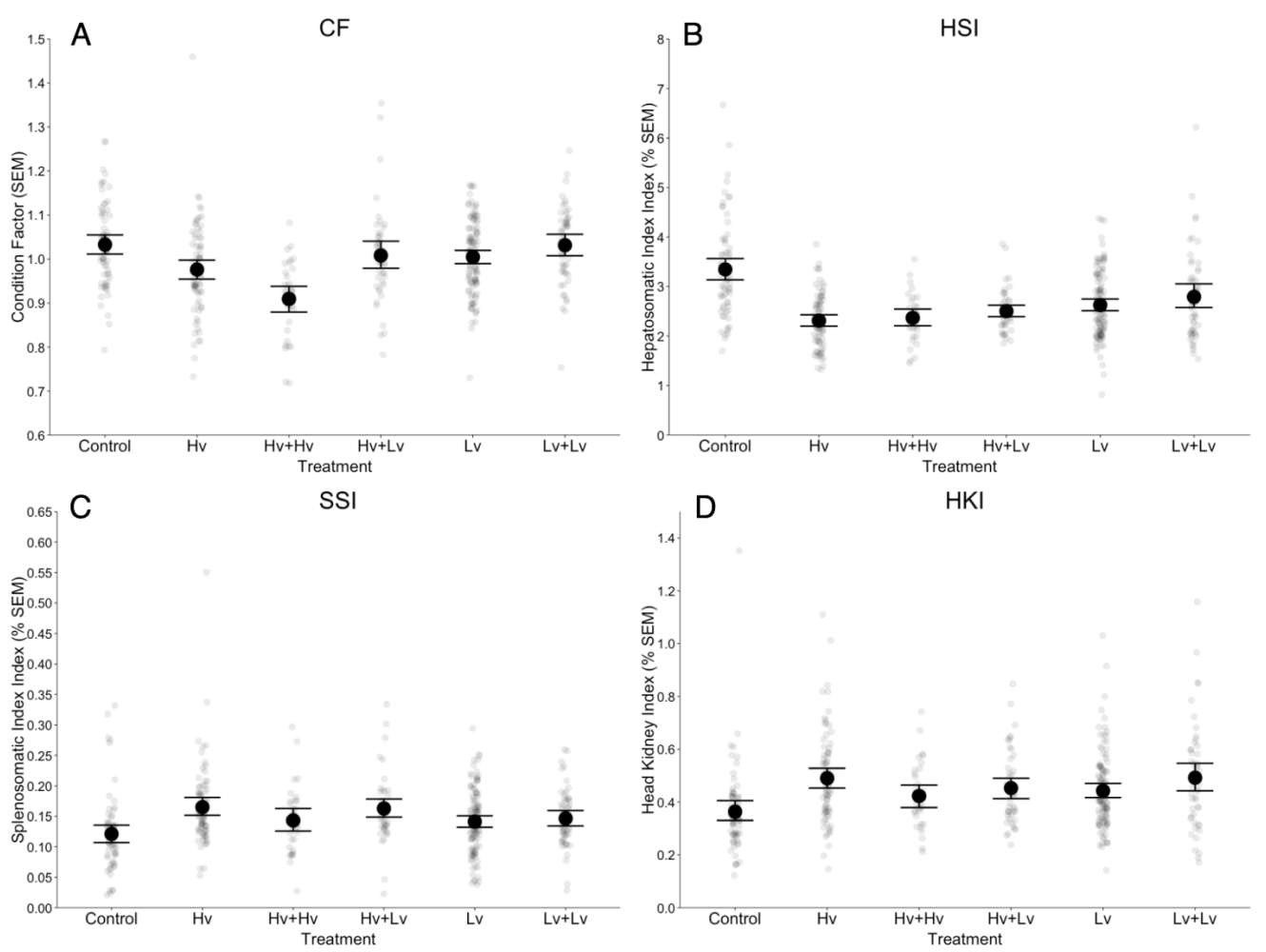

Figure IV-4: Variation in fish condition and immune parameters

The means for different indices for fish nutrional condition and immune system parameters are shown as means ±SEM (Control: N = 72, Hv: N = 80, Hv+Hv: N = 34, Hv+Lv: N = 47, Lv: N = 113, Lv+Lv: N = 53).

A: Mean condition factor (CF)

The CF was only lower in fish that were exclusively infected with Hv parasites (Hv and Hv+Hv) compared to unexposed control fish.

B: Mean hepatosomatic index (HSI)

The HSI was lower in all infected treatment groups compared to unexposed control fish.

C: Mean splenosomatic index (SSI)

The HKI was higher in all infected treatment groups compared to unexposed control fish, expect for fish infected with two Hv parasites (Hv+Hv)

D: Mean head kidney index (HKI)

The HKI was higher in all infected treatment groups compared to unexposed control fish.

There was a significant effect of the treatment on the SSI ($F_{5,392} = 6.369$, $p < 0.0001$) and *a post hoc* Tukey HSD test showed significant differences between treatment groups: the SSI was significantly higher ($p < 0.05$) in all treatment groups compared to the unexposed control fish apart from the Hv+Hv double infections (Figure IV-4C). Interestingly, although this treatment had the highest parasite burden it was the only treatment that was not significantly different from the unexposed control fish ($p > 0.05$).

The head kidney index (HKI) was calculated to describe the ratio between head kidney weight and fish body weight. An increase in head kidney size is assumed to be a consequence of an elevated immune response (Press & Evensen, 1999), possibly also due to a parasite infection.

There was a significant effect of the treatment on the HKI ($F_{5,392} = 11.355$, $p < 0.0001$) and *a post hoc* Tukey HSD test showed that it was significantly higher in all infection treatments compared to unexposed control fish ($p < 0.01$) (Figure IV-4D). Even though there were striking differences in parasite burden between the treatments, all infections stimulated the immune system to a similar, non-significantly differentiated level, except for the comparison between fish singly infected with one Hv or on Lv parasite. Fish infected with one Hv parasite had a significant higher HKI than fish infected with one Lv parasite ($p < 0.05$).

Discussion

Most of our understanding about the effect of competition between co-infecting parasites on virulence is derived from observational studies of natural populations or based on theoretical approaches (Alizon *et al*., 2013; Baalen & Sabelis, 1995; Brown *et al.*, 2002; Frank, 1992). By using the *Schistocephalus*-stickleback model system in an experimental approach we have revealed that intraspecific interaction can have significant but varying effects on individual parasite virulence. This was made possible by our macroparasite system, which contrary to many studies on microparasites, allowed us to study both individual parasite virulence (discrete Parasite Index dPI) and overall virulence of co-infecting parasites (total Parasite Index tPI).

Virulence Model

There are several lines of evidence that indicate that the virulence of an individual parasite is determined by a combination of intrinsic factors and the production of both specific and unspecific public goods (SI Figure S2).

The heterologous infection had an intermediate total parasite burden. Strikingly, this is the result of an increased virulence in the Lv parasite combined with a decrease in virulence for the Hv parasite compared to their performance in homologous infections. This is contrary to a scenario where solely intrinsic factors (SI Figure S2a) or only specific public goods (SI Figure S2c) determine the virulence of a parasite in co-infections, where we would have expected the Hv and Lv parasite to reach the same size as in their respective homologous infections. The most likely explanation for the intermediate total parasite burden is the sharing of public goods. However, the fact that the Hv parasite remained significantly larger than the Lv parasite points to the fact that these public goods must be both specific and unspecific (SI Figure S2d). If virulence was determined by unspecific public goods alone, either parasite should benefit equally from the common pool and therefore both individuals should have reached the same size (SI Figure S2b).

The interactions between parasites of the same virulence type also have significant effects on individual virulence. In both homologous double infections, individual parasite virulence was significantly reduced. In the Hv+Hv double infection the total parasite burden was still higher than in Hv single infections, which indicates that all other treatment groups were not limited by host resources in general. However, this effect was not seen with Lv parasites. It is not clear whether this is due to a self-limitation to avoid overexploitation or the inability to successfully exploit more host resources. These results still fit our model of specific and unspecific public goods that, together with an intrinsic ability, determine the virulence of the parasites (SI Figure S2).

In the case of homologous double infections, the parasites probably reached their specific growth ceiling. The significantly lower condition factor (CF) in the Hv+Hv double infections confirms that the Hv parasites were able to access more resources than in other treatments. This is evidence that all other treatments were not limited by host resources. Considering the CF was not significantly different between Lv single and Lv+Lv double infections this is indicative of an intrinsic host exploitation ceiling for the Lv parasite. However this does not mean there is no intrinsic host exploitation ceiling for the more virulent parasite, it may just be higher.

Virulence model mechanisms

In *S. solidus* virulence seems to be a plastic trait that is not only shaped by the interaction of the parasite with the host's immune system, but also by intraspecific interactions among co-infecting parasites. Those intraspecific interactions are mediated by the production of specific and unspecific public goods that are most likely excretory/secretory products that modify the immediate environment of the parasite, i.e. the fish body cavity (Hewitson *et al.*, 2009). In cestodes like *S. solidus* both exchanges with the host and food intake require transport through the outer tegument. Thus public goods that could increase host nutrients availability, as well as induce immune manipulation would affect the body cavity environment of co-infecting parasites and be an important factor in this interaction (Pedersen & Fenton, 2007).

This is consistent with Jäger and Schjørring (2006) who have shown that in sequential infection the younger *S. solidus* plerocercoids had a fitness advantage over the older 'pioneering' parasites. In fact, second infecting plerocercoids had a higher survival rate and discrete parasite index than the first infecting plerocercoid. Moreover, the fitness advantage was even larger if the two worms were genetically related. This suggests a cost to inducing host immune manipulation and growth, that this investment can beneficiate another conspecific (public goods) and is also more profitable if the two parasites are closely related (specific public goods).

There is evidence for immune manipulation by *S. solidus* (Scharsack *et al*., 2004) and recently it has been found that this immune manipulation may be mediated by *S. solidus* excretory/secretory products (Scharsack *et al*., 2013). *In vitro* immune modulations differ between several *S. solidus* populations, highlighting the specificity of the immune manipulation and virulence of different *S. solidus* strains, which could be part of a specific public goods system (Franke *et al*., 2014).

Host manipulation

Two types of immune manipulation could be beneficial for *S. solidus*. The most obvious one would be immune suppression/evasion, which is a well-known effect of chronic helminth infections (Maizels & Balic, 2004). A less intuitive form of immune manipulation would be to evoke an immune stimulation. The immunopathology phenomenon induced by an acute immune response could weaken the host and cause tissue damages that could be advantageous for the parasite (Long & Boots, 2011). An elevated immune response could increase the amount of nutrients available in the body cavity by mobilizing cells to migrate to the parasites.

To understand if immune manipulation is present in our case and if it is expressed as immune suppression or immune stimulation, we can use the splenosomatic index (SSI) and head kidney index (HKI). Infected fish showed significant elevations in these indices compared to unexposed control fish, which indicates immune stimulation in response to the infection. Interestingly, the variation between the different infection types was rather low. From this we can conclude that the infection itself triggers an immune response that leads to an elevation of relative spleen and head kidney weight, but that the intensity of the immune stimulation does not increase with parasite virulence. One major exception occurred in this pattern: the SSI of Hv+Hv infected fish was not different from the control and lower than the SSI of fish from the other infections treatments. This could either be because two Hv parasites limit their immune manipulation to avoid over-exploitation or, the fish is already over-exploited and incapable of mounting a costly immune response.

Host resources availability

To determine the accessibility of the second type of public goods, the nutrients, we can use the CF and hepatosomatic index (HSI), which show the nutritional status of the fish host. Both showed a reduction when the fish were infected, although this was less prominent in the CF. Only Hv parasites (in single or homologous double infections) significantly reduced the CF of fish. It is therefore possible, that the condition factor is directly linked to parasite size here. For the HSI we see a similar pattern as for our immune-relevant indices: all infection treatments showed a significantly lower HSI than unexposed control fish, while the variation between the different infection treatments was rather low.

All these measurements indicate that an infection per se has a large impact on the host's immune system and its nutritional status, but they do not explain the variation in parasite virulence.

Parasite communication

Parasites must find a balance between manipulation of the immune system and avoiding being targeted by it or overexploiting the host. This can be either mediated through a feedback loop between each parasite and the host (Pedersen & Fenton, 2007) or by direct parasite-parasite interactions.

Parasites could monitor the impact of host manipulation and exploitation by the level of immune suppression and/or resource availability they benefit from. Such a feedback loop would lead to a reduction in host exploitation when an intrinsic ceiling is reached. Another

solution for virulence regulation would be directed by some kind of communication between co-infecting parasites as is often the case in bacterial infections (Diggle *et al.*, 2007). Evolution could have favored such communication mechanisms, as in multiple infections overexploitation can rapidly and dramatically reduce their chances of transmission and therefore their fitness (Parker *et al.*, 2003).

Conclusion

Our results show that intraspecific competition can directly alter the virulence of a parasite. In the case of co-infecting parasites of contrasting virulence, the more virulent parasite suffers from a decrease in virulence, while the less virulent parasite can achieve a higher virulence than without the presence of a competitor. This suggests, that the virulence of an individual parasite, that does not replicate in this intermediate host, is a plastic trait and is not only determined by the interaction of the parasite with the host's immune system, but also by the interaction among co-infecting conspecifics. These results have important implications on an evolutionary level, where low virulent parasites can be favored in the presence of high virulent parasites, which pay a cost for the "free-loading" conspecific. As this can also influence the epidemics of parasitic diseases in animals and humans (Petney & Andrews, 1998), more experimental work should be conducted on multiple infections involving different strains. Additionally, our results cannot fully distinguish between a direct interaction of two parasites and an indirect interaction via feedback loops with the host. Future studies on the transcriptome of both parasite and host will hopefully help to answer this question.

Methods

Model parasite

Schistocephalus solidus is a trophically transmitted pseudophyllidean cestode with a complex life cycle involving three different hosts. *S. solidus* reproduces sexually in the intestine of its final host, a piscivorous bird. The parasite's eggs are released with the bird's feces into the water, where free-swimming larvae can hatch and infect the first intermediate host, cyclopoid copepods. When the second intermediate host, a three-spined stickleback (*Gasterosteus aculeatus*), ingests an infected copepod, the procercoid stage can establish in the fish body

cavity and develop into a plerocercoid stage. *S. solidus* can infect various bird and copepod species but it is specific for its second intermediate host and can only infect three-spined stickleback. It undergoes most of its growth in the three-spined stickleback, and the size of the parasite is directly correlated to the damages caused to its fish host (Bagamian *et al*., 2004), as well as to its reproductive success in its final bird host via fecundity and mate-choice decisions (Lüscher & Wedekind, 2002; Schärer *et al*., 2001; Wedekind *et al*., 1998). Therefore the size reached by the parasite in the three-spined stickleback can serve as a proxy for parasite virulence and parasite fitness.

S. solidus from a German (Neustädter Binnenwasser, 54° 06' 41" N, 10° 48' 33" E) and a Norwegian population (Skogseitvatnet, 60° 14' 38" N, 05° 54' 51" E) were used. Parasites were specifically chosen from these populations, as earlier experiments have shown dramatic differences in their virulence (Kalbe & Jakobsen, in prep). Yearly field surveys of the Norwegian and the German populations have shown contrasting epidemiology of the *S. solidus* infection in the two systems. The Norwegian system has a relatively high prevalence (usually >40% on average), where multiple *S. solidus* infections are fairly common, whereas the German system present a relatively low prevalence (usually <1%) and low/no occurrence of multiple infections (unpublished data). Moreover, experimental data had repeatedly shown that *S. solidus* strains exhibit consistent virulence type, with some strains being always more virulent then others when infecting different fish populations. In this context the Norwegian parasite strain has a consistently relatively higher virulence compared to the German strain, and the Norwegian fish exhibit a higher resistance than the German host (Kalbe & Jakobsen, in prep). Additionally, helminths like *S. solidus* that reproduce in the intestine and disperse through the feces of migratory birds, are likely to experience gene flow between large geographically spread meta-populations. Thus, the probability of a high-virulent and low-virulent strain to interact within the same fish host is conceivable.

The German and Norwegian tapeworms were respectively classified as low virulent strain (Lv) and high virulent strain (Hv).

Experimental exposure

The experiment was conducted in two independent rounds in two separate years. In each year, three different fish families and three different parasite families from each parasite population were used in a full factorial design to minimize genotype specific effects that would mask the treatment effects. Only in the Hv+Lv heterologous double infection we created three Lv and Hv families independent combinations out of all the possible ones. We

ended up having three different family combinations for each treatment groups in both rounds.

In total six lab-bred families of *S. solidus* from each, the Hv population and the Lv population were used to experimentally expose six lab-bred families of three-spined sticklebacks originating from the lake Großer Plöner See (Germany, 54°09'21"N, 10°25'50"E).

Following the *in vitro* system developed by Smyth (Smyth, 1946) and modified by Wedekind (1997), the eggs of the *S. solidus* families were incubated for 21 days at 20°C in the dark, before being placed to light to trigger coracidia hatching. Lab-cultured copepods (*Macrocyclops albidus*) were then singly exposed to *S. solidus*. The copepods were starved for one day before exposure and afterwards fed every second day *ad libitum* with *Paramecium caudatum*. Starting 6 days post-exposure, copepods were microscopically checked for the presence of procercoids. At 16 days post-exposure, to limit developmental variability between procercoids and increase the chance of infection (Benesh & Hafer, 2012), each fish was individually exposed to two, one or none infected copepod to create six treatment groups (Figure IV-1). After 24 hours the fish were grouped into 16L rearing tanks with an average number of 12 fish per tank.

The treatment groups aimed at producing co-infections by performing simultaneous double exposure to two high virulent parasites (Hv+Hv), two low virulent parasites (Lv+Lv) or to a heterologous combination of the two (Hv+Lv). As control groups, single exposure to one high virulent parasite (Hv), one low virulent parasite (Lv) or to none (control) were performed (Figure IV-1).

To exclude confounding effects through genetic relatedness of the parasites, two different *S. solidus* families were always used for homologous double infections; in heterologous double infections the two parasites were unrelated by nature. In total this resulted in six different combinations for each double exposure treatment.

Dissection

Eight weeks post exposure fish were killed with an overdose of MS222 (tricaine methanesulfonate, 1mg/ml), given a cervical incision and subsequently dissected to record standard length, weight, sex and weights of key internal organs (head kidneys, spleen, liver, gonads and the body kidney). If the fish was infected, the number of plerocercoids and the individual parasites weight were noted.

The gonad weight was subtracted from the total fish weight to reduce the effect of differences in the level of sexual maturation and the following indices were calculated: total parasite

index (tPI, with tPI = (total parasite weight [g] / fish weight [g])*100), condition factor (CF, calculated after Frischknecht, 1993), hepatosomatic index (HSI, with HIS = (liver weight [g] / fish weight [g])*100), splenosomatic index (SSI, with SSI = (spleen weight [g] / fish weight[g])*100) and head kidney index (HKI, with HKI = (head kidney weight [g] / fish weight [g])*100). The relative parasite weight or discrete parasite index (dPI) describes the relationship between the weight of one individual parasite and the fish weight, discriminating between two parasites in the same fish and was calculated as dPI = (individual parasite weight [g] / fish weight [g])*100.

To genotype the parasites and thereby determine the identity of the successfully infecting parasite(s), a tissue sample of each parasite was collected.

Microsatellite typing

For all parasites we collected from fish in this experiment the DNA was extracted using the DNeasy Kit from Qiagen. Five different microsatellite loci (Binz *et al*., 2000) were used to determine origin and parasite family for each individual.

Statistical Analyses

As the experiment was run over two different years, the factor year was included as a random factor in all analyses.

For infection rate analysis the dataset analysed with a linear mixed model using both year and sex as random factors, due to known differences in infection rates between male and female sticklebacks (Reimchen & Nosil, 2001).

The dataset used for the analysis of the total parasite index (tPI) included all infected fish. For the condition factor (CF), hepatosomatic index (HSI), splenosomatic index (SSI) and head kidney index (HKI), the dataset included all infected fish and the control fish (not exposed to a parasite). For the discrete parasite index (dPI) the dataset included all infected fish and each individual parasite's data (family, origin, treatment, parasite weight).

The response variables were transformed to achieve normal distribution, if necessary (log transformation for HSI and SSI, square root transformation for HKI).

All statistics were carried out using R (R 2.12.2 (R Development Core Team; www.r-project.org)).

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Conclusion & Perspectives

Adaptations of parasites to their hosts are complex and diverse. One mechanism that allows close coevolution between one host and one parasite is a high degree of host specificity in the parasite. Infecting only a very low number or, as in the case of *Schistocephalus*, only one specific host species can be beneficial for the parasite, if it leads to an efficient mechanism to manipulate the host's immune system and behaviour.

In my thesis I have shown that the high host specificity of *Schistocephalus* is likely due to the stickleback immune system being able to target the incompatible *Schistocephalus* species in the body cavity, and subsequently, eliminate them within the first two weeks of the infection process (Chapter I). The mechanism by which this high specificity is maintained is still unclear. One possibility are specific carbohydrate structures on the parasite surface which have been shown to be correlated to infectivity and growth of *S. solidus* (Hammerschmidt & Kurtz, 2005b) in three-spined sticklebacks. These structures also vary between different sibships and may play a crucial role in the successful crossing of the mucosal barrier in the fish gut. This may also explain why *S. solidus* seemed to be unable to reach the body cavity of *Danio rerio*. This result demonstrates the parasite's ability to still reach the body cavity of a non-specific host, but not a non-related fish species, indicating that the migration through the fish's gastrointestinal tract may also underlie specific constraints, possibly mediated through the surface carbohydrate composition.

A possibility on how fish might discriminate between different *Schistocephalus* species may be through the ability of parasites to cloak themselves with host molecules and prevent recognition by the fish immune system (molecular mimicry). If the parasites are able to mask themselves only in specific host-parasite combinations, this may also explain why *S. solidus* plerocercoids in the body cavity of the incompatible host *Pungitius pungitius* seem to be encapsulated and disintegrated.

Future studies could target the presence of host-specific molecules on the surface of the parasite or the parasite-specific surface carbohydrates in both species and their hybrids to shed light on the mechanism behind this specificity.

Even though it is known that *Schistocephalus* manipulates the stickleback's immune response (Franke *et al*., 2014; Scharsack *et al*., 2004; Scharsack *et al*., 2013), this does not allow a second, incompatible parasite to take advantage of the situation and successfully infect the

stickleback. This may be further indication that molecular mimicry is more important than the ability to directly manipulate the host immune system for successful establishment.

We could also show that the two different parasite species – even though they have a deep lineage divergence – can still produce viable hybrid offspring (Chapter II). Surprisingly, hybrids are able to infect both stickleback species just as well as the parental species infect their specific host. These results indicate that host specificity in *Schistocephalus* is a co-dominant trait, most likely something that provides the parasite with the possibility to escape the stickleback immune response. This could be through the ability of molecular mimicry which is effective for both stickleback species, allowing the parasites to evade an immune response. Since the hybrids did not suffer from obvious fitness disadvantages (except for a higher rate of selfing and a lower hatching rate), it is unclear, why this high degree of host specificity evolved and is maintained in this system. Therefore *Schistocephalus* spp. and their stickleback hosts are an ideal system to study host specificity in more detail.
Such studies could include the genetic basis of host specificity, how many and which genes are involved in this trait and how these evolved in the different *Schistocephalus* species. A genome-wide scan for genes responsible for host specificity could be performed to identify the genomic regions responsible and these data could be compared among the different species to investigate the origin and evolution of host specificity in this taxon. For this purpose, it would also be interesting to cross other *Schistocephalus* species and see if hybridization is possible among more distantly related species and whether this also results in host range expansion. Much less is known about *S. cotti* and *S. nemachili*, including their relatedness and host range. At least in *S. cotti* it was suggested that this species may use several different intermediate hosts (Dechtiar *et al*., 1966; Hoffman, 1999; Margolis & Arthur, 1979; Nagasawa *et al*., 1989; Sterud, 1999), even though it has not been confirmed, whether these individuals all belong to the same species (Chubb *et al*., 2006).

We furthermore investigated possible prezygotic mechanisms that could reinforce speciation in the two parasite lineages (Chapter III). These results show that i) the two parasites hybridize not only in the *in vitro* breeding system, but also in a natural host, ii) there are no barriers that prevent hybridization in sympatric parasite populations and iii) *Schistocephalus* prefers parasites of the different species as mates over conspecifics.
All these results point in the direction that hybridization would be beneficial for the parasite – or – that there would have been a selection pressure on the parasite to not diverge into

separate species each with this high degree of host specificity. Postzygotic mechanisms that could prevent gene flow between the two species can still exist; these mechanisms have not yet been investigated. Therefore, the question why we see such a high degree of host specificity remains.

One possibility could be the close adaptation between parasite and stickleback immune system. As we could show, the incompatible parasite can still establish within the "wrong" host, but is eliminated early in the infection which supports this hypothesis. This raises the question whether host specificity is costly. If so, hybrids that expand their host range may not grow as large as their parental species in their specific host. Nonetheless, the hybrids do not suffer in terms of reduced growth in both stickleback hosts, which would have been an indicator that host specificity is indeed costly in the second intermediate host.

More work needs to be done to understand, why the deep lineage divergence between *S. solidus* and *S. pungitii* exists and how it is maintained. This should especially focus on postzygotic mechanisms, such as hybrid infertility or F2 hybrid breakdown. As our results also indicate a lower hybridization rate between the two species that originate from a sympatric population, more work should be conducted that could reveal costs for host specificity. Furthermore, molecular tools would allow scanning natural populations of *Schistocephalus* spp. for interspecific gene flow and possible natural hybridization events.

It is now widely accepted that host specificity can play an important role in the speciation process of parasites (Duffy *et al*. 2007; Henry *et al*., 2008). Speciation in parasites can either occur through host-switching (Barker, 1991; Clayton, *et al*. 1996) or co-speciation of hosts and parasites (Hafner & Nadler, 1988). The latter possibility often leads to congruent phylogenies of hosts and parasites, a phenomenon known as Fahrenholz's rule (Eichler, 1948). Not enough is known about the speciation events in *Schistocephalus* spp. and if host specificity was the driving force in the process. Nevertheless, co-speciation seems to be a possibility for *S. solidus* and *S. pungitii* (Nishimura *et al*., 2011). Other members of the genus *Schistocephalus* infect stone loach (*Barbatula barbatula*, infected by *S. nemachili* (Dubinina, 1959)) and bullheads (*Cottus* spp., infected by *S. cotti* (Chubb *et al*., 2006)). As these fish species are only distantly related to sticklebacks, host-switching events may have also been important in the speciation process of *Schistocephalu*s spp. More work on the phylogenies of *Schistocephalus* spp. and their corresponding hosts is needed to answer these questions.

Even within the species of *S. solidus* differences regarding virulence in the second intermediate host are known from different parasite strains. In the last part of this thesis

(Chapter IV), we investigated how intraspecific interactions influence the virulence of *S. solidus* in the three-spined stickleback. We could show that in heterologous co-infections involving one high and one low virulent parasite, the low virulent parasite benefits from the presence of a high virulent conspecific by growing larger than in single infections. This had negative consequences for the high-virulent parasite that exhibited reduced growth in the presence of a low virulent parasite when compared to homologous double infections with two high virulent parasites. These results indicate that the intrinsic ability of a parasite to exploit and manipulate a host can differ between strains, but also that there are certain public goods that can be shared either between conspecifics of the same strain or among parasites of different strains resulting in some form of communication between the parasites. These parasite-parasite interactions do not only alter individual parasite virulence but also have the potential to impact host-parasite coevolution dynamics on the long run.

The evolution and determinants of virulence are extensively studied, but there is still more that needs to be done. *Schistocephalus* could be an ideal model system to study the genetics of virulence. As this is a trait that can be accurately and quantitatively measured (i.e. parasite size) in this system, this would offer many possibilities for future research. Crossing a high virulent and a low virulent strain could reveal a first insight into the genetics underlying virulence in this system and inbred F2 offspring could then be used to analyse the quantitative genetics of virulence in *S. solidus*. The recent development of molecular tools to access and analyse gene expression in *S. solidus* also provides the opportunity to investigate the interactions between *S. solidus* and its host and among co-infecting individuals on a transcriptomic level.

Summarized, in my thesis I show that the adaptation of a parasite to a host can be complex, and differ between and within a certain species. The ability to exploit a certain host successfully is not only specific to a species, but also specific to a certain strain which can lead to co-speciation of hosts and parasites. In natural communities, the situation is even more complex, as hosts are usually infected with multiple parasites, not only of different strains but also with different parasites species that interact with each other.

Author contributions

Chapter I:

Henrich T & Kalbe M: Host specificity in the *Schistocephalus*-stickleback system.

Author contributions: TH and MK designed the study, TH performed the experiments, analysed the data and wrote the manuscript.

Chapter II:

Henrich T, Benesh DP, Kalbe M: Hybridization between two cestode species and its consequences for intermediate host range. Parasites & Vectors 2013, 6:33

Author contributions: MK conceived the study. TH and MK performed the experiment, TH analysed the data and wrote the manuscript. DPB performed the analysis for the evaluation of the infection bias and helped with the statistics and the manuscript. All authors read and approved the final manuscript.

Chapter III:

Henrich T & Kalbe M: Prezygotic barriers to hybridization in *Schistocephalus* spp.?

Author contributions: TH and MK designed and performed the experiments, TH analysed the data and wrote the manuscript.

Chapter IV:

Henrich T[§], Erin NI[§], Phelps L, Kalbe M: Intraspecific competition alters the virulence of the parasite *Schistocephalus solidus* in three-spined stickleback

([§]equal contribution)

Author contributions: TH, NIE and MK designed research, TH, NIE, LP and MK performed research, TH and LP analyzed data, NIE interpreted the phenotypic results, TH drafted the paper, TH, NIE, LP and MK wrote the paper.

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List of publications

During the time of my doctoral studies I have authored the following publication, which is not included in my thesis:

Henrich, T; Hafer, N; Mobley, KB (2014): Effects of VIE tagging and partial tissue sampling on the immune response of three-spined stickleback *Gasterosteus aculeatus***.** Journal of Fish Biology, 85(3): 965-971

Eidesstattliche Erklärung

Hiermit erkläre ich, dass ich die vorliegende Dissertation mit dem Titel

Host specificity and adaptation of *Schistocephalus* to its stickleback hosts

selbstständig, mit der Unterstützung meiner Betreuer, verfasst habe. Ich habe keine anderen als die angegebenen Hilfsmittel und Quellen verwendet und die Arbeit unter Einhaltung der Regeln guter wissenschaftlicher Praxis der Deutschen Forschungsgemeinschaft erstellt.
Diese Arbeit wurde an keiner anderen Stelle im Rahmen eines Prüfungsverfahrens vorgelegt und ist mein bisher erstes und einziges Promotionsverfahren.

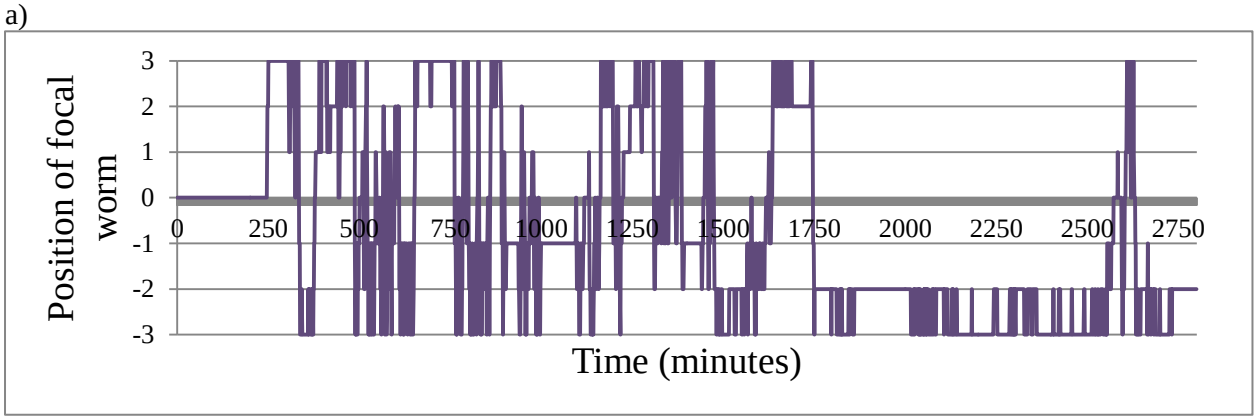
Kapitel II dieser Arbeit wurde in der wissenschaftlichen Fachzeitschrift „Parasites & Vectors“ veröffentlicht. Die Koautoren aller Kapitel finden sich zu Beginn des jeweiligen Kapitels in der Autorenliste. Der Anteil der Autoren an den Manuskripten wird im Abschnitt „Author contributions“ erläutert.

Plön, im November 2014 Tina Henrich

Appendix

Appendix Chapter III

a)



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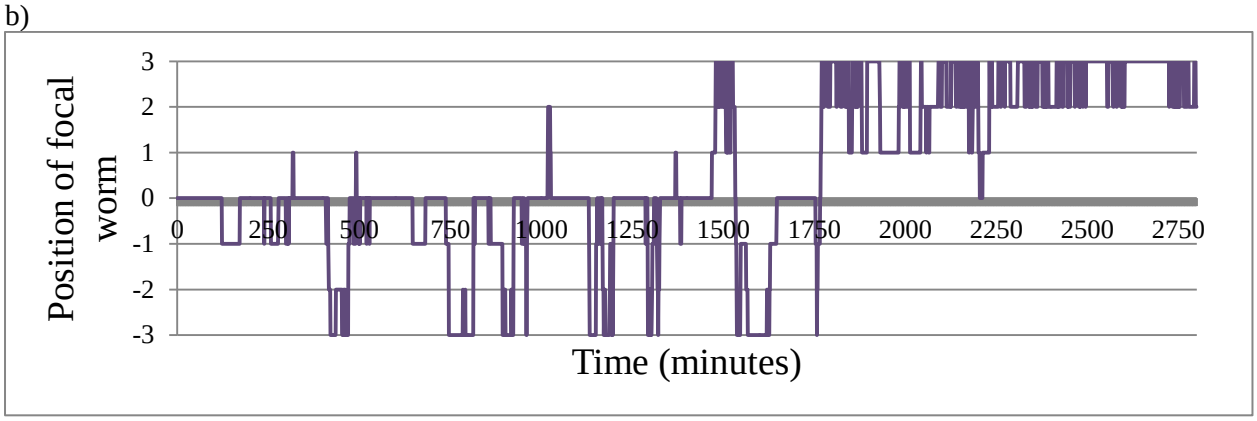

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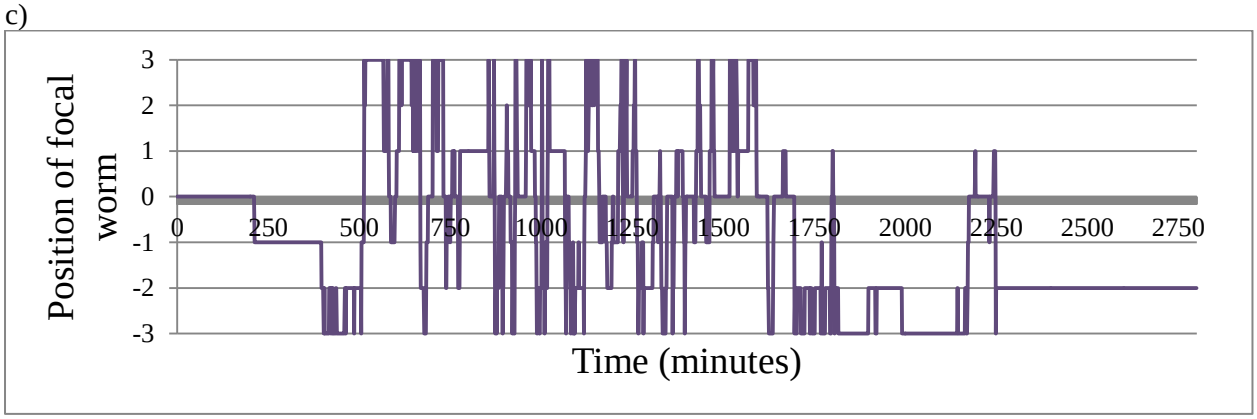

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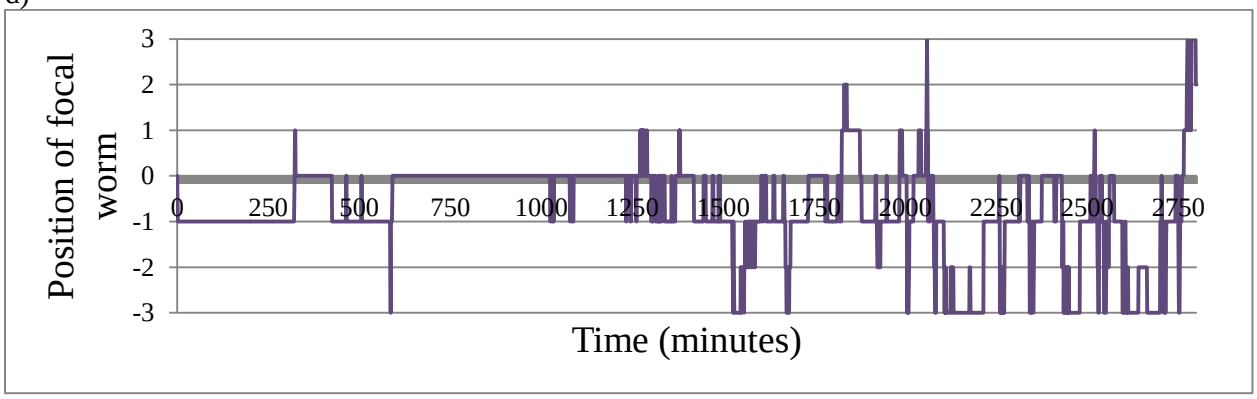
Position of focal worm
3
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750
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1250
1500
1750
2000
2250
2500
2750
Time (minutes)

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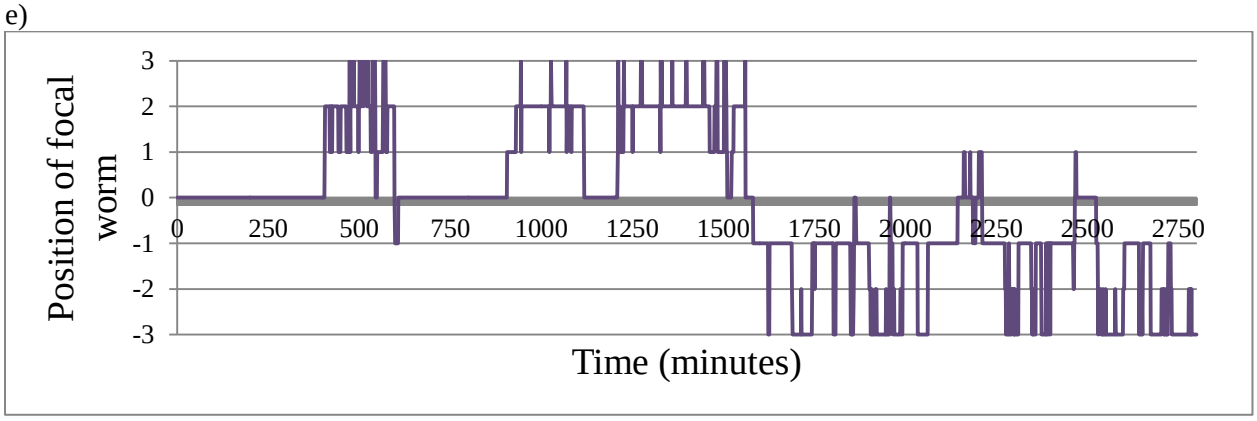
Position of focal worm
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Time (minutes)

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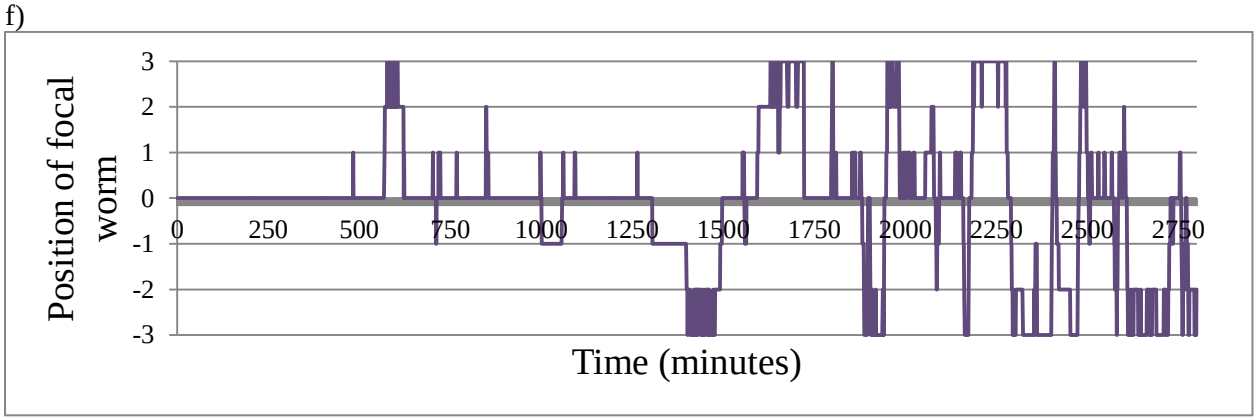
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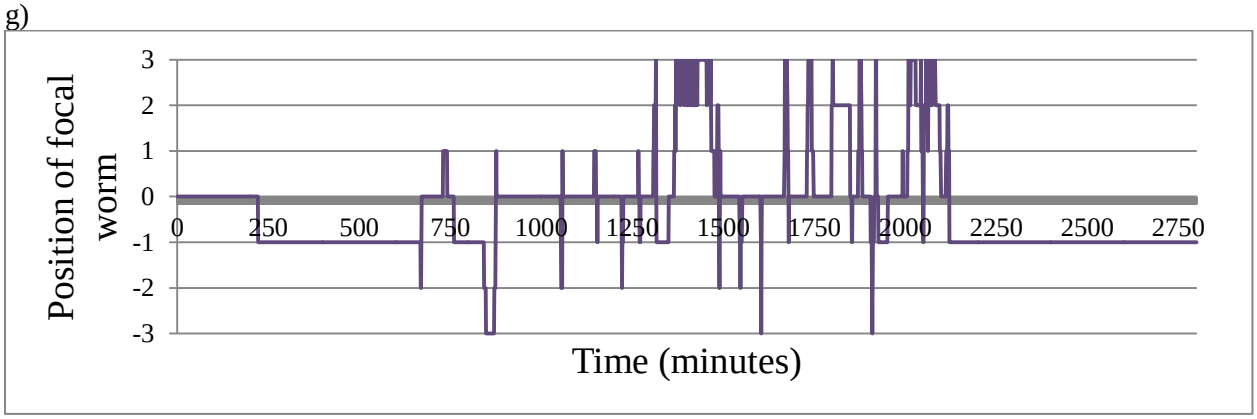
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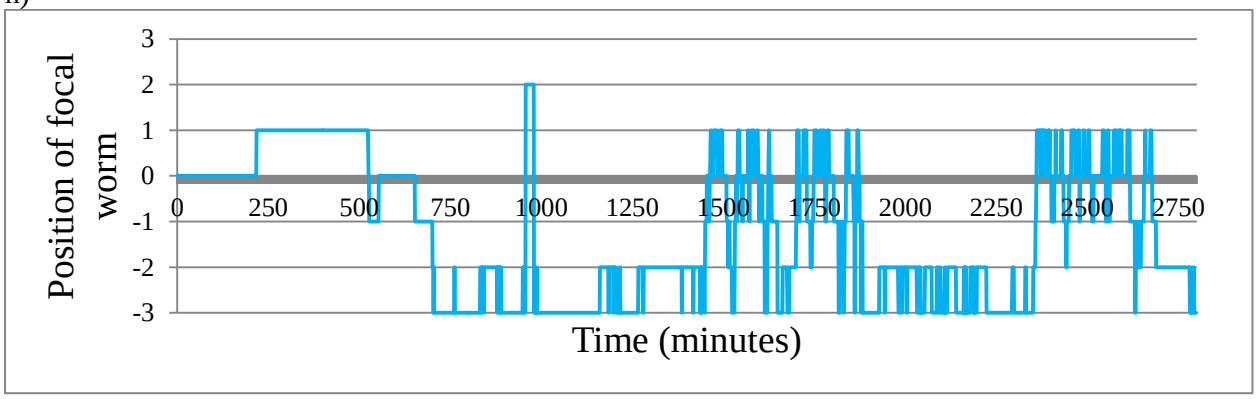
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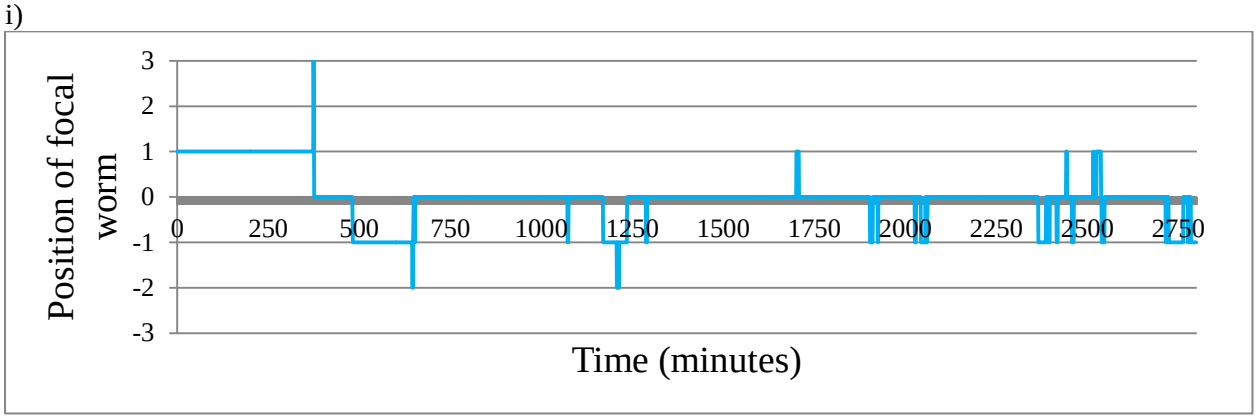
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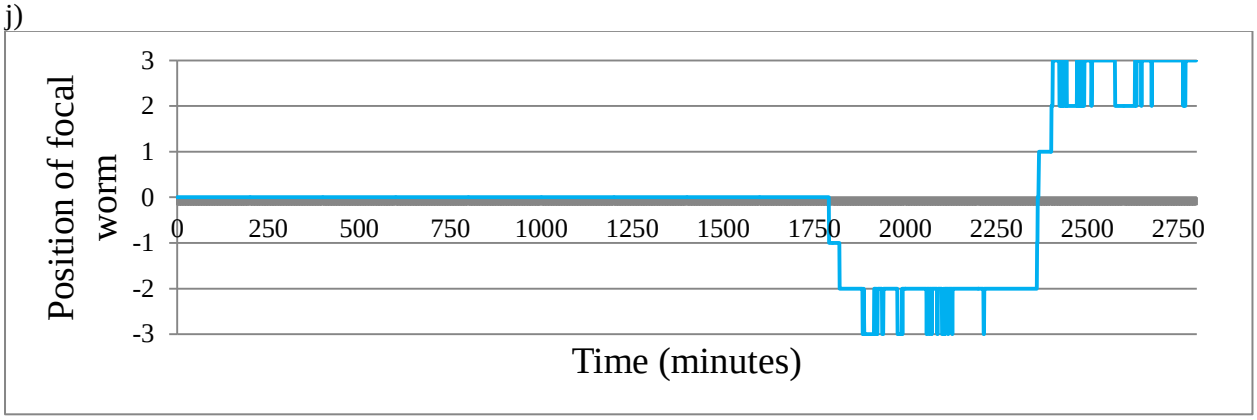
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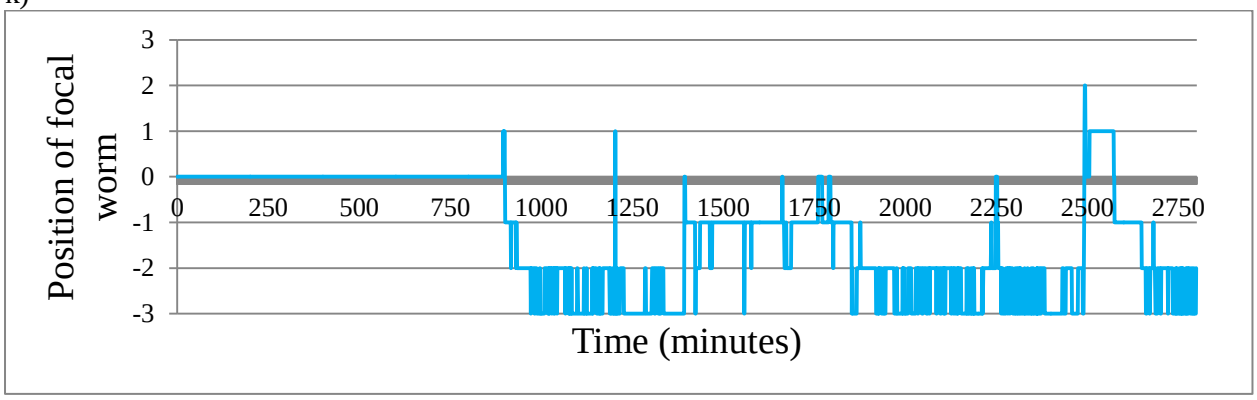
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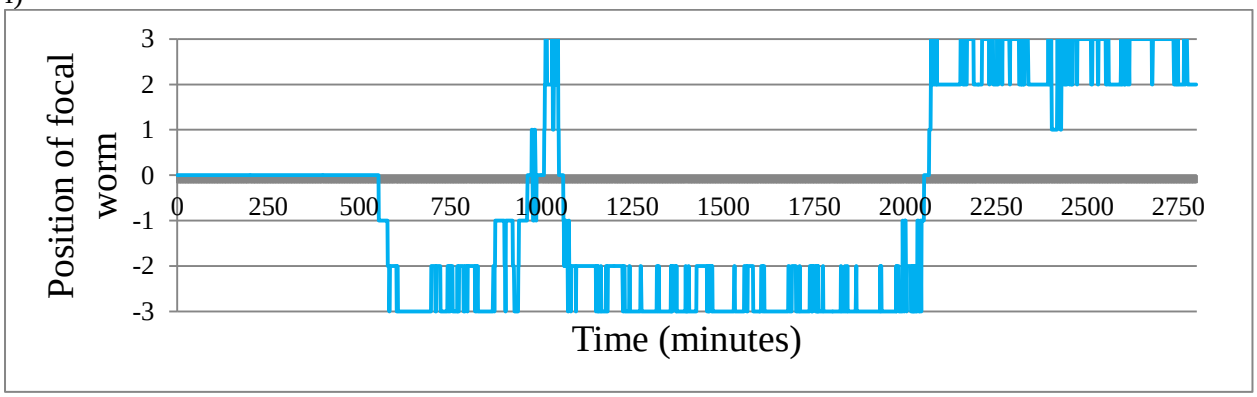
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Time (minutes)

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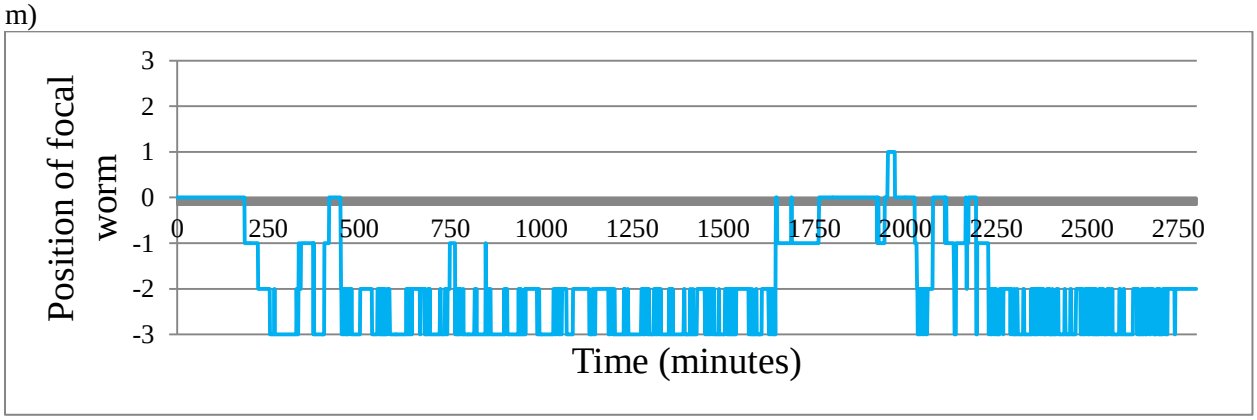
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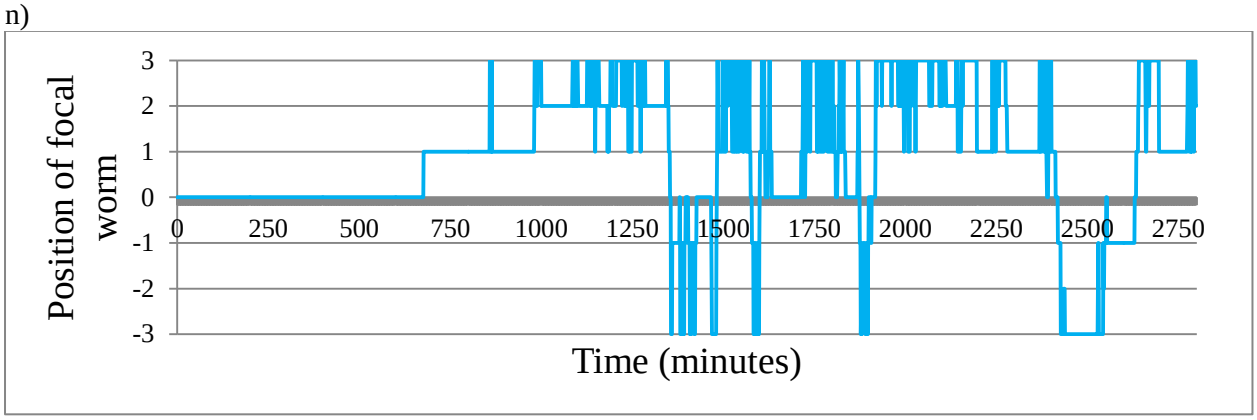
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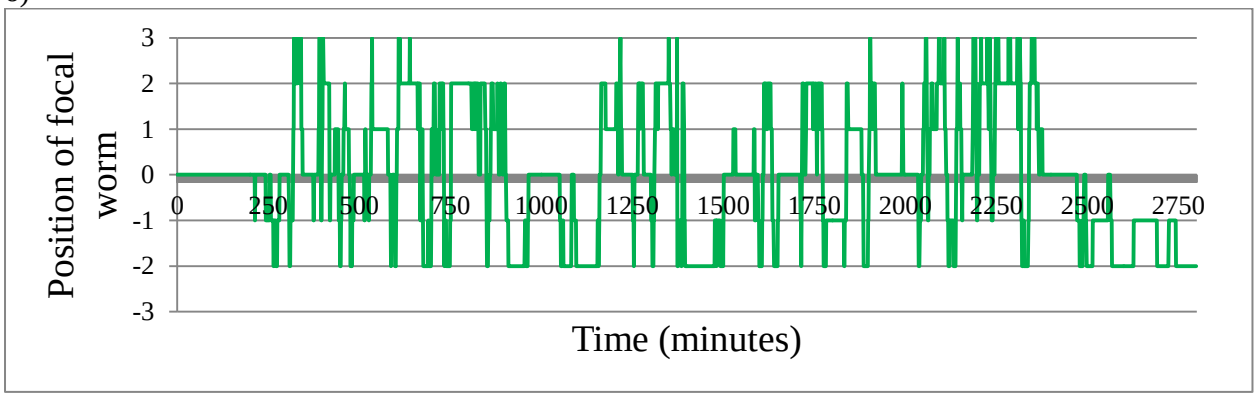
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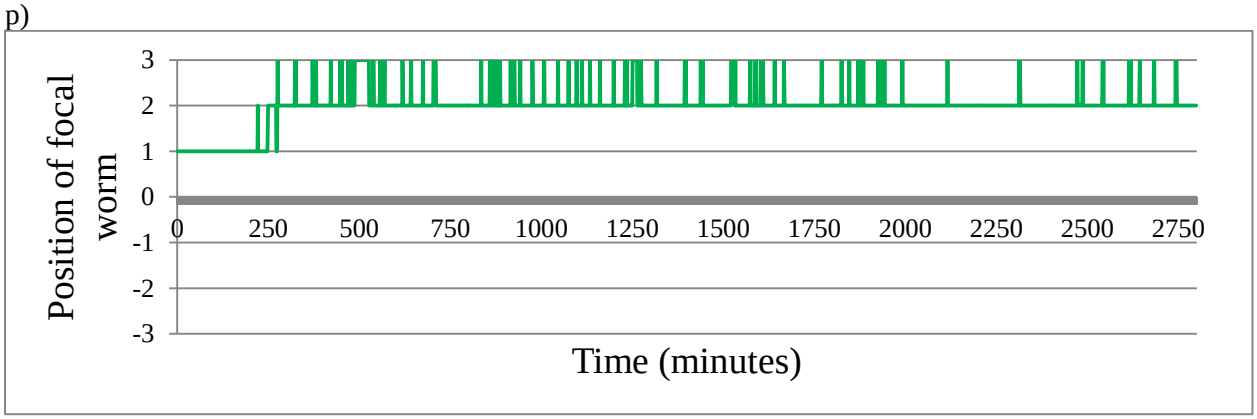
Position of focal worm
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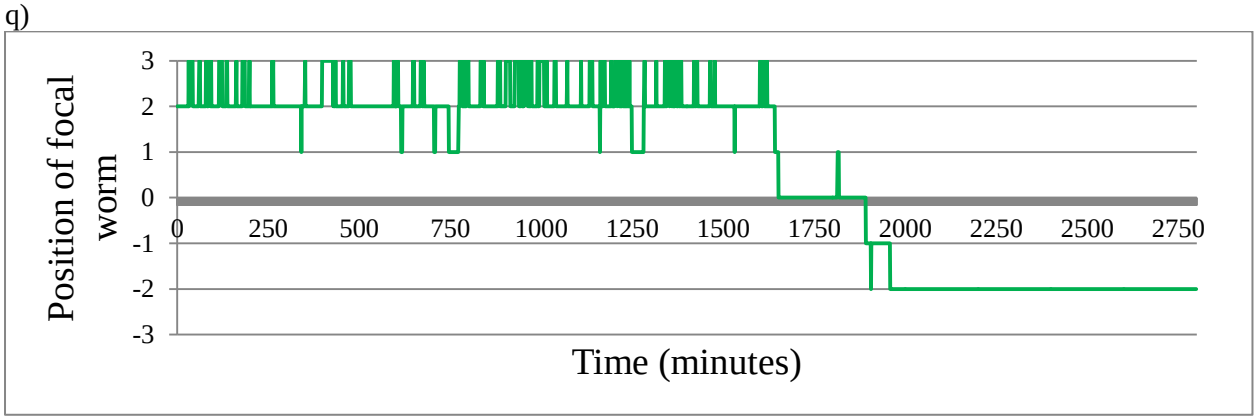
Position of focal worm
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Time (minutes)

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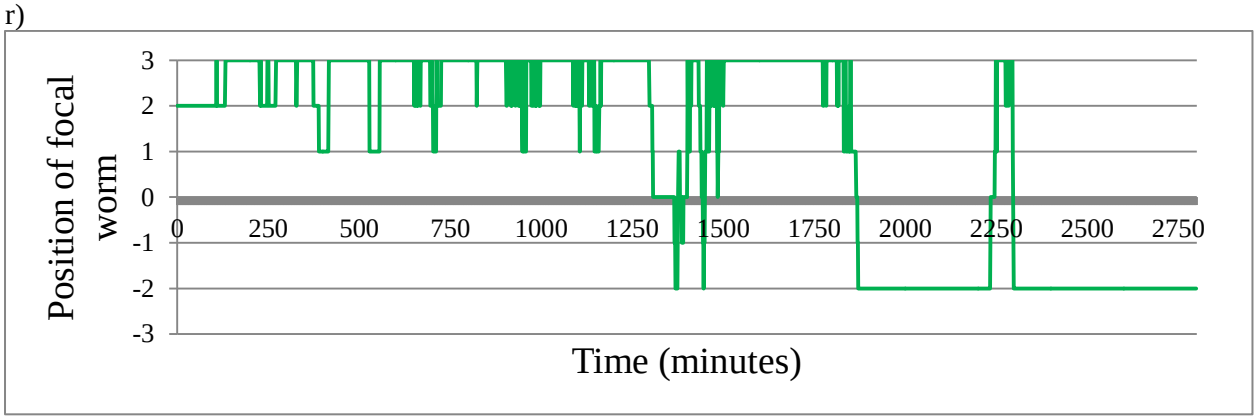
Position of focal worm
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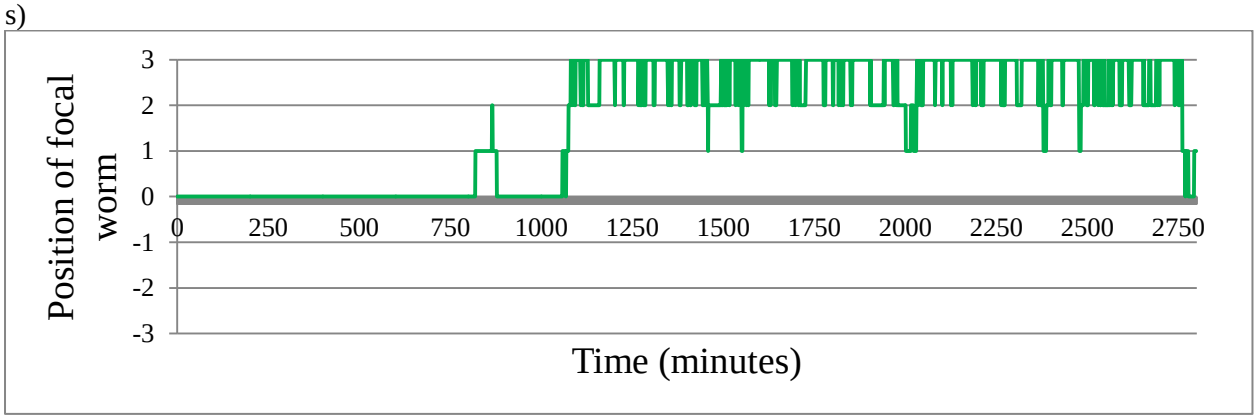
Position of focal worm
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Time (minutes)

Figure S1: Individual profiles of the position of each focal worm in the mate choice experiment

S. pungitii was the focal worm in trials a-g (purple), *S. solidus* in trials h-n (blue) and the controls are trials m-s (green). Positive scores for the position indicate the location of the conspecific, negative scores indicate the position of the worm from a different species or empty compartments (for controls only).

Appendix Chapter IV

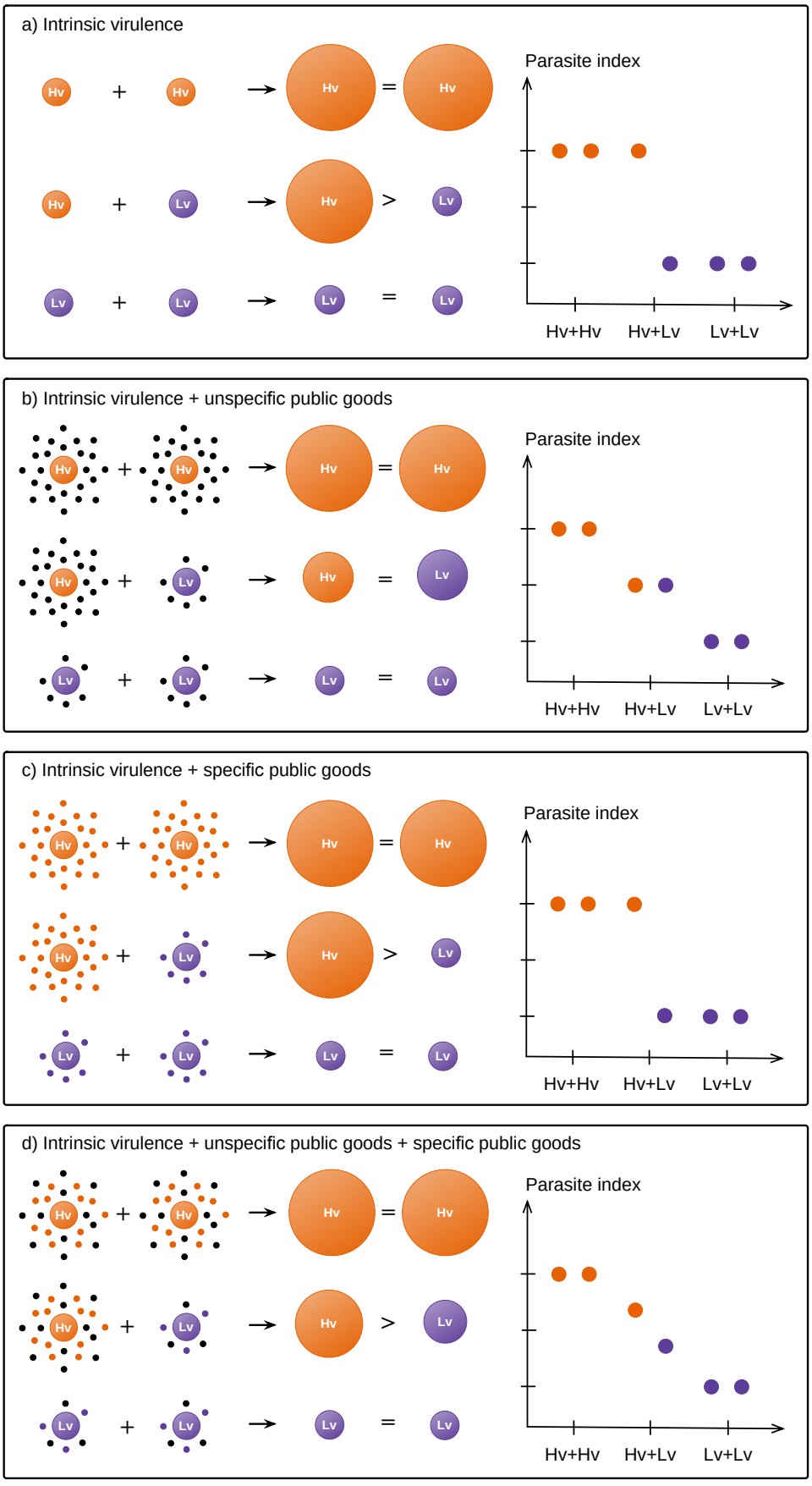

Figure S2: Virulence models for co-infecting parasite

The effect of different virulence models combining intrinsic factor and the production of public goods (proportional to the intrinsic virulence level) on the discrete parasite index of High virulent (Hv) and Low virulent (Lv) parasites in double homologous infection (Hv+Hv and Lv+Lv) or heterologous infection (Hv+Lv).

a) Intrinsic virulence

If virulence is an intrinsic factor, the parasite index is solely determined by the virulence type (Hv or Lv) and is independent of the virulence level of a co-infecting parasite.

b) Intrinsic virulence + unspecific public goods

If virulence is a combination of intrinsic factor and the production of unspecific public goods (black dots), all the resources produced by the co-infecting parasites are available and equally shared by both. In this case, the parasite index of heterologous co-infecting parasites is the same and is intermediate to the one reach in homologous infection.

c) Intrinsic virulence + specific public goods

If virulence is a combination of intrinsic factor and the production of specific goods (colored dots), the public goods produced by a virulence type are only available for this specific virulence type and cannot be use by a heterologous co-infecting parasite. This mimics the effect of a solely intrinsic virulence so the parasite index is independent of the virulence level of the co-infecting parasite.

d) Intrinsic virulence + unspecific public goods + specific public goods

If virulence is a combination of intrinsic factor and the production of both unspecific (black dots) and specific public goods (colored dots), only a part of the public goods produced by a virulence type are available to a heterologous co-infecting parasite. In this case, a Lv parasite will beneficiate sharing a host with a Hv parasite which is producing more unspecific public goods. The two parasites will reach an intermediate total parasite index but the Hv parasite will still reach a higher discrete parasite index than its co-infecting Lv parasite, thanks to the production of specific public goods. These predictions correspond to the results of our experiment.